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The Genetics of Lithium-Induced Adverse Drug Reactions in Bipolar Disorder Patients: A Pilot Study

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List of Abbreviations

°C	degrees Celsius
μl	microlitre(s)
μM	micromolar
3'	3 prime
5'	5 prime
95% CI	95% confidence interval
A	adenine
ADR	adverse drug reaction
AKT	protein kinase B
AKT1	V-akt murine thymoma viral oncogene homolog 1
AMPA	alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionate
APOE	Apolipoprotein E
AQP2	aquaporin 2
ARRB2	β-arrestin 2
Asp	aspartic acid
<i>BDNF</i>	brain-derived neurotrophic factor
BLAST	Basic Local Alignment Search Tool
bp	base pair(s)
BPD	Bipolar Disorder
C	cytosine
Ca ²⁺	calcium
cAMP	cyclic adenosine monophosphate
CASR	calcium-sensing receptor
CBZ	carbamazepine
ConLiGen	Consortium on Lithium Genetics
CREB	cyclic AMP response element-binding protein
CYP	cytochrome P450
D'	linkage disequilibrium coefficient
DALY	disability-adjusted life years
ddNTPs	dideoxynucleotide triphosphates
dH ₂ O	distilled water
DISC1	disrupted in schizophrenia 1
DNA	deoxyribonucleic acid

dNTPs	deoxynucleotide triphosphates
DRD1	dopamine receptor D1
DRD2	dopamine D2 receptor
DSM-IV	Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition
EDTA	ethylenediaminetetra-acetic acid
ENaC	epithelial sodium channel
<i>et al.</i>	<i>et alii</i>
EtBr	ethidium bromide
<i>ExoI</i>	Exonuclease I
F	forward
FAM	C-carboxyfluorescein
<i>FYN</i>	Src-family tyrosine kinases
g	gram
G	guanine
Glu	glutamic acid
Gly	glycine
GPCRs	G-protein-coupled receptors
GRIA2	glutamate/ AMPA receptor
<i>GSK3</i>	Glycogen synthase kinase 3
GWAS	genome-wide association studies
GxE	gene environment interaction
HIF-1	hypoxia-induced factor-1
HPT	hyperparathyroidism
HREC	Human Research Ethics Committee
HWE	Hardy-Weinberg Equilibrium
IDT	Integrated DNA Technologies
IMPA1	inositol monophosphatase 1
IMPase	inositol monophosphatase
INPP1	inositol polyphosphate 1-phosphatase
IP3	inositol phosphate
IPP	inositol 1-polyphosphate phosphatase
iU	international unit
kb	kilobase pairs

I	litre
LD	linkage disequilibrium
LTD	long-term depression
LTG	lamotrigine
LTP	long-term potentiation
M	molar
MDD	major depressive disorder
mEq	milliequivalents
mg	milligram
Mg ²⁺	magnesium
MGB	minor groove binder
min	minute(s)
ml	millilitre(s)
mM	millimolar
mmol	millimole
mRNA	messenger RNA
MSRI	magnetic resonance spectroscopy imaging
MWM	molecular weight marker
N	Any nucleotide
Na ⁺ -K ⁺ -ATPase	sodium-potassium-transporting ATPase
NCBI	National Centre for Biotechnology Information
NDI	nephrogenic diabetes insipidus
ng	nanogram(s)
NMDA	N-methyl-D-aspartate
NMDAR	N-methyl-D-aspartate receptor
NRFU	normalised relative fluorescent units
OR	odds ratio
P13Ks	PI3 kinases
PCR	polymerase chain reaction
PD	Parkinson's disease
PD	pharmacodynamic
pg	picogram(s)
PGx	pharmacogenetics or pharmacogenomics
PI	phosphatidyl inositol

PK	pharmacokinetic
pmol	picomoles
PP2A	protein phosphatase 2A
PPAR	Peroxisome proliferator-activated receptor
PPARGC1a	PPAR gamma, co-activator 1 alpha
PTH	parathyroid hormone
R	reverse
r^2	correlation coefficient
RE	restriction endonuclease
RFLP	restriction fragment length polymorphism
RFU	relative fluorescence units
RNA	ribonucleic acid
ROX	6-Carboxy-X-rhodamine
rpm	revolutions per minute
s	second(s)
SASH	South African Stress and Health Study
SCID	Structured Clinical Interview for DSM Disorders
SD	standard deviation
Ser	Serine
SNP	single nucleotide polymorphism
T	thymine
T_a	annealing temperature
<i>Taq</i>	<i>Thermus aquaticus</i>
TBE	tris-borate-EDTA buffer
TD	Tardive dyskinesia
TE	tris EDTA
TH	thyroid hormones
T_m	melting temperature
TPH	tryptophan hydroxylase
Tris	tris(hydroxymethyl)aminomethane
TSH	thyroid stimulating hormone
TSH	thyroid stimulating hormone
U	unit
UCT	University of Cape Town

UTR	untranslated region
UV	ultraviolet
V	volts
VIC	2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein
VPA	valproic acid
WHO	World Health Organisation
WMH	WHO World Mental Health
Y	pyrimidine (C or T)

Abstract

Lithium is regarded as the first-line pharmacotherapy for the treatment of acute mood episodes, suicide prevention and prophylactic treatment in patients with bipolar disorder (BPD). Response to lithium has a strong genetic component and lithium-responders have an increased frequency of BPD among their family members. Lithium has a narrow therapeutic index and 75-90% of patients on long-term lithium treatment experience one or more side effects, such as weight gain, cognitive decline and skin problems, amongst at least 20 side effects.

The research project is immersed in a larger project on the genetics of bipolar disorder, in which a large number of individuals in families have been investigated over several years. The present pilot study explored whether single nucleotide polymorphisms (SNPs) within *GSK3B*, *AKT1*, *ARRB2*, *GRIA2* and *PPARGC1A* could be associated with the incidence and severity of lithium-induced side effects. One hundred and five patients with a diagnosis of BPD type I and a history of treatment with lithium were genotyped for ten SNPs within the candidate genes using SNaPshot PCR, TaqMan SNP genotyping and RFLP analysis. A questionnaire related to the twenty most common side effects, as well as treatment history, was conducted with all patients.

Results show an association between the cognitive side effects of lithium treatment, such as memory and concentration difficulties, and the splice-variant rs6438552 in *GSK3B*, an association between weight gain and rs1130233 in *AKT1*, as well as an association between skin side effects, related to lithium treatment, and the coding variant rs8192678, in *PPARGC1A*. Chi-squared analysis further indicated associations between polydipsia and body aches with variants in *PPARGC1A*, and associations of nausea with rs10138227 in *AKT1* and indigestion with rs4302506 in *GRIA2*. These four associations did not remain significant when covariates were taken into account using logistic regression analysis.

Although exploratory, these results indicate that at least some of the genes investigated in this study may play a role in influencing interindividual susceptibility to lithium-induced side effects, and may be clinically important in the treatment of BPD patients. This sets the stage for a larger scale study aimed at understanding appropriate treatment for this debilitating and often fatal disorder.

Chapter 1. Introduction

1.1. Features and Characteristics of Bipolar Disorder

Bipolar Disorder (BPD) is a chronic psychiatric illness. The more severe form, Type 1, which has a world-wide prevalence of 0.6 – 1 % (Merikangas et al., 2007; Merikangas et al., 2011) is characterised by recurrent episodes of mania and depression. Psychotic features such as delusions and hallucinations may also be a feature (American Psychiatric Association, 1994). The affected individual usually experiences a decline in quality of life, productivity, and longevity, as well as having greater difficulty in sustaining interpersonal relationships and maintaining employment, compared to those without mood disorders (Calabrese et al., 2003a).

BPD is a major public health problem with severe socioeconomic implications. In 2009, the total economic burden of BPD in the USA was estimated at US \$151 billion, the cost of which is attributed to treatment, hospitalisation, misdiagnosis, and consequent productivity loss (Hirschfeld and Vornik, 2005; Dilsaver, 2011). Nearly one third of patients admits to attempting suicide at least once and between 10% and 20% of patients successfully commit suicide (Müller-Oerlinghausen et al., 2002).

The diagnosis of BPD is based exclusively on clinical symptoms as the molecular pathology has not been discovered (Phillips and Kupfer, 2013). Due to the symptomatic overlap between BPD and other psychiatric disorders, such as Unipolar Depression and Schizophrenia, an accurate clinical diagnosis is difficult to make (Maier et al., 2005). Patients are often misdiagnosed or diagnoses are delayed and their disease is not effectively treated and managed.

The aetiology of BPD is still poorly understood, but the disease is known to involve genetic and epigenetic (Abdolmaleky et al., 2006; D'Addario et al., 2012) mechanisms as well as environmental factors (Alloy et al., 2005). Genetic variation amongst affected individuals has been investigated in order to determine the factors underlying the pathophysiology of BPD. The majority of genetic studies suggest that BPD has a high level of genetic heterogeneity and a substantial polygenic component (Craddock et al., 1995). Meta-analyses have found consistent supportive evidence for linkage to a few potential BPD susceptibility loci especially on chromosomes 6q, 8q, 13q and 22q (Badner and Gershon, 2002; Segurado et al., 2003; McQueen et al., 2005).

Polymorphisms in a variety of candidate genes have been tested for association with BPD, but the small number that appear to be associated show odds ratios of 1.1–1.3, which is consistent with a polygenic basis for the disease (Nurnberger et al., 2008). Several genome-wide association studies (GWAS) have been conducted, but few associations have been replicated in more than one study (Sklar et al., 2011). Apart from the locus heterogeneity model of BPD, it is hypothesised that it is the regulatory elements, and not necessarily the coding or structural component of genes associated with psychiatric disorders that may be disrupted (Perkins et al., 2004).

As no biological markers for BPD currently exist, neuro-imaging studies have been conducted to examine structural changes in brain regions involved in mood and behaviour (Adler et al., 2006; Scherk et al., 2008). Meta-analysis has shown that BPD patients have reduced cerebellar volumes compared to healthy controls (De Peri et al., 2012).

1.2. Diagnosis of BPD

The diagnosis of BPD is hindered by several factors, including the symptomatic overlap with other psychiatric disorders, as well as comorbidity with other illnesses. BPD patients often have another Axis I (or II) disorder which can complicate diagnosis and treatment (McElroy et al., 2001). These disorders include substance and alcohol abuse, anxiety and panic disorders, and personality disorder.

According to the Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV), BPD type I is marked by the occurrence of one or more Manic Episodes or Mixed Episodes (American Psychiatric Association, 1994). One or more Major Depressive Episodes is also often present. Patients that experience a milder form of mania (hypomania), without psychotic symptoms and without harmful behaviour to themselves, or others, are diagnosed as having BPD type II.

1.2.1. Description of Mania

A manic state is an immense disturbance of emotion that is characterised by euphoria, overactivity, flight of ideas, and positive psychomotor signs (American Psychiatric Association, 1994). When untreated, a manic phase can last months or years (Belmaker, 2004). Manic episodes are accompanied by an increase in mental and physical energy, which may be seen in the form of pressured speech, sexual promiscuity, racing thoughts, distractibility, psychomotor agitation and a decreased need for sleep (American Psychiatric Association, 1994).

A manic state is not always stable and it may be interrupted by tearfulness, or intense irritability, hostility, or even aggressiveness (Akiskal, 1995). This is termed a mixed episode, as manic and depressive symptoms are observed simultaneously. The loss of self-control and inhibition experienced during mania often leads to damaged relationships and financial debt, which only serves to worsen the depressive episode following mania (Goodwin and Jamison, 2007).

1.2.2. Description of Depression

BPD patients with bipolar depression experience psychological feelings of worthlessness, inappropriate guilt, and have the inability to concentrate or think clearly. This is accompanied by a number of physical disturbances, including insomnia or hypersomnia, weight gain or loss, psychomotor agitation or retardation, fatigue and lassitude (American Psychiatric Association, 1994). Psychotic features may also present that often have themes of worthlessness, sinfulness and persecution (Akiskal, 1995).

1.3. Bipolar Disorder in South Africa

In spite of the high burden of BPD and other psychiatric conditions in the world, few epidemiological needs assessment surveys have been carried out in sub-Saharan Africa (Ovuga et al., 2005). Due to several factors, the South African population is at high risk for psychiatric disorders (Kessler et al., 2007). A major concern is the history of political violence and victimisation of the Apartheid era, as well as the increasingly high rates of violent crimes and violence against women and children in the post-Apartheid era (Dunkle et al., 2004; Dinan et al., 2004). In addition, it has been reported that the harsh economic circumstances and the risk of HIV/AIDS could contribute to higher rates of emotional distress (Seedat and Stein, 2000; Hughes et al., 2006). The available evidence suggests high and ever increasing suicide rates (Flisher et al., 2004) and high levels of alcohol abuse in South Africa (Parry et al., 2005).

The South African Stress and Health Study (SASH) was conducted as part of the WHO World Mental Health (WMH) Survey Initiative from January 2002 to June 2004 (Kessler et al., 2007). The study found the prevalence of psychiatric disorders to be notably higher in SASH than in a number of other country-wide surveys. Markedly, the prevalence of substance abuse in South Africa, i.e. 5.8%, was at least twice that in other WMH countries, except for the Ukraine, where it is 6.3% (Williams et al., 2008).

BPD was not evaluated in isolation, but the 12-month prevalence of all mood disorders was estimated to be 5% (Herman et al., 2009). In the Western Cape province of South Africa, the prevalence of BPD is estimated to be 1%, and it is the most common reason for admission of female patients to Acute Psychiatric Services (Kleintjes et al., 2006).

1.4. Treatment of Bipolar Disorder: Mood stabilisers, Anticonvulsants, Antipsychotics and Antidepressants

The long-term treatment of BPD is through several classes of drugs, such as anticonvulsants, antipsychotics (especially the atypical antipsychotics) and lithium, collectively termed “mood stabilisers”. Although they differ in chemical structure and mechanism of action, all these drugs commonly act as prophylactic agents in the prevention of illness recurrence. A mood stabiliser should ideally be effective in the treatment of acute manic symptoms, acute depressive symptoms, as well as in the prevention of manic and depressive symptoms (Bauer and Mitchner, 2004).

Lithium is regarded as the first-line treatment for prevention of further episodes in BPD1 (APA, 2002; Lewis et al., 2010; Yatham et al., 2013). Among anticonvulsants, valproic acid (VPA), lamotrigine (LTG) and carbamazepine (CBZ) have been shown to be effective in the long-term management of BPD (Muzina et al., 2005; Bowden, 2009). VPA, CBZ, as well as lithium can be used in the treatment of acute mania, but it has been suggested that these drugs work too slowly in the majority of patients with acute mania (Verdoux et al., 1996). LTG on the other hand, is especially effective in the treatment of bipolar depression (Passmore et al., 2003).

Antipsychotics are very effective in the treatment of acute mania (Biederman et al., 1979), although long-term use is avoided due to the risk of developing tardive dyskinesia. As these drugs can be administered parenterally and have a rapid effect, they are especially critical for acute treatment in violent or psychotic patients (Belmaker, 2004). The atypical antipsychotics, which cause less severe extrapyramidal side effects, are also effective in the treatment of mania and may additionally have a lower risk of inducing depression than the classic antipsychotics. Their utility in the long-term management of BPD is, however, controversial as patients may have increased risk for developing severe adverse drug reactions while on maintenance treatment (Vieta and Goikolea, 2005).

Bipolar depression is generally treated with tricyclic antidepressants, selective serotonin-reuptake inhibitors, and monoamine oxidase inhibitors (Kusumakar, 2002), as in patients with unipolar depression. However, more care should be taken in bipolar patients as antidepressants may induce mania. Antidepressants are, therefore, avoided in patients with a history of dangerous manic episodes, in which the life of the patient, his family or his job were endangered (Belmaker, 2004).

The “Standard Treatment Guidelines and Essential Drugs List for South Africa” provides standard treatment guidelines for the treatment of BPD in South Africa (The National Department of Health, 2012). A summary of these drugs, the drug type and the aspects of treatment it is used for are given in Table 1.1.

Table 1.1: Drugs included in the “Standard Treatment Guidelines and Essential Drugs List for South Africa” for the treatment of bipolar disorder

Drug	Treatment	Drug type
Lithium	Maintenance, Depressive episodes	Mood stabiliser
Sodium Valproate	Maintenance, Mixed Episodes	Anticonvulsant
Fluoxetine	Depressive episodes	Antidepressant
Carbamazepine	Mixed episode	Anticonvulsant
Haloperidol	Acute managements, Psychosis	Antipsychotic
Benzodiazepines	Acute management	Anticonvulsant
Chlorpromazine	Psychosis	Antipsychotic
FTD; FZD; ZD	Psychosis	Antipsychotic
Clozapine	Psychosis	Atypical antipsychotics
Risperidone	Psychosis	Atypical antipsychotics
Sulpiride	Psychosis	Atypical antipsychotics
FTD -Flupenthixol decanoate, FZD -Fluphenazine decanoate, ZD -Zuclopenthixol decanoate		

Combination treatment, or polypharmacy, is the rule rather than the exception in BPD patients and most patients are on a combination of lithium or VPA, an antidepressant and/or an atypical antipsychotic (with a median total of three medications) (Post et al., 2010). In spite of the multiple therapeutic tools used in the treatment of BPD, a significant number of patients may still not respond to treatment, even after consecutive trials with various drugs.

Drug combinations can cause neurotoxicity, which likely results from different drug interaction mechanisms (Boeker et al., 2011). A better understanding of mechanisms of drug action, and genetic factors involved in drug response, is therefore crucial to maximise treatment efficacy and to prevent dangerous side effects.

Pharmacogenetic or pharmacogenomic (PGx) studies aim to identify genetic variants that lead to interindividual differences in drug response and susceptibility to adverse drug reactions (ADRs). PGx studies can be divided into pharmacokinetic (PK) and pharmacodynamic (PD) approaches, where the former pertains to genetic variants that effect drug metabolism and the latter to genetic variants that have effects on drug targets, including downstream signalling pathways (Evans and Johnson, 2001).

The prospect of pharmacogenetically determining effective personalised treatment is especially appealing in BPD, where patients are often treated with multiple drugs and where effective treatment is determined on a trial and error basis. Understanding the factors influencing response to treatment does not only hold the promise of better treatment for patients, but can also aid in elucidating the underlying genetic cause of BPD.

The present study investigates lithium treatment in patients with BPD type I, with a focus on genetic factors that may influence the prevalence and severity of ADRs in these patients. Current knowledge on lithium's mechanism of action and lithium-induced ADRs are discussed in Chapters 2 and 3, respectively. The materials and methods, results and discussion of the study are presented in Chapters 4 to 6.

Chapter 2. Lithium Treatment

Lithium is a monovalent cation that was first used in the clinical treatment of BPD in the 1950's (Cade, 1949). Clinically, it remains the first line treatment, and is administered as a salt in carbonate or citrate formulations. Slow release and immediate release medications are available with the former producing relatively more stable plasma lithium levels. Due to the possibility of toxicity developing at high levels, lithium levels are carefully monitored and maintained at a therapeutic serum concentration of 0.6-1.2mEq/L.

Numerous control and open trials have shown lithium to reduce episode recurrences by at least 30% (Carney and Goodwin, 2005; Coryell, 2009) and at optimal dosing, it reduces 50% of the recurrences (Calabrese et al., 2003b; Bowden et al., 2003). Lithium, further, has high efficacy in reducing the number of suicide attempts in patients with recurrent major affective disorders (Rihmer, 2011; Cipriani et al., 2013).

Response to lithium has a strong genetic component and patients who respond to lithium generally have an increased frequency of BPD among their family members (Grof et al., 1994; Grof et al., 2002; Cruceanu et al., 2008; Cruceanu et al., 2009). Lithium-responders tend to have euphoric mania, good inter-episode recovery and less comorbidity with other psychiatric illness, whereas patients with irritable mania or mixed states tend to respond better to anticonvulsants (Calabrese et al., 1996; Berghofer et al., 2008). It has therefore been suggested that lithium-responders have a distinct form of BPD from non-responders (Swann et al., 1997; Passmore et al., 2003). Some aspects of the pharmacokinetic and pharmacodynamic properties of lithium will be discussed, as well as the major findings of lithium pharmacogenetic studies.

2.1. Lithium Pharmacokinetics

Lithium is not metabolised and approximately 95% is renally excreted (Keck Jr and McElroy, 2002). Lithium has a low therapeutic index, and toxicity can occur just above the therapeutic range, necessitating monitoring of plasma drug concentration (Keck Jr and McElroy, 2002). A further caution is that neurotoxicity may occur even when serum lithium levels are within therapeutic range. The risk for developing neurotoxicity is, however, increased due to additional factors, such as dehydration, systemic infection, hyperthermia, the use of other drugs and renal impairment (Roy et al., 1998; Lang and Davis, 2002).

Lithium may require six to ten days to reach steady serum concentration, as it moves slowly through the extracellular compartment. Its distribution in organs is near uniform and similar to the distribution of sodium in the body (Groleau, 1994). Following glomerular filtration within the kidneys, at least 70% of the drug is reabsorbed by the renal tubules, due to a competition of lithium with sodium in the sodium-proton antiporter (reviewed in Battle et al., 2008).

The absorption of lithium into cells occurs mostly through a voltage-sensitive sodium channel, which has a permeability near or equal to that for sodium (Armett and Ritchie, 1963; Vasilyev et al., 2002). Efflux occurs through sodium-lithium countertransport, the exact pathway of which is not clear (Sarkadi et al., 1978). Some studies, however, show that the pump responsible for sodium-lithium countertransport is likely to be the sodium-hydrogen exchange protein (Serrani et al., 2000; Ng et al., 2000).

Peak lithium levels in the brain are reached after approximately 24 hours (Chen et al., 2004). The half-life of lithium in the brain is 28 hours, compared to 16 hours in the serum. A high-resolution whole brain 3D Lithium-7 magnetic resonance spectroscopy imaging (MRSI) study on human BPD patient brains have demonstrated that lithium is not evenly distributed throughout the brain, but is concentrated in specific brain regions, especially at higher lithium serum concentrations (Lee et al., 2012). Some of these regions are part of the anterior limbic network that maintains emotional homeostasis and lithium's effectiveness may be due to it specifically targeting these areas (Mega et al., 1997; Strakowski et al., 2004).

2.2. Lithium Pharmacodynamics

Despite numerous studies showing the effectiveness of lithium in the treatment of especially acute mania, the exact mechanism of lithium action has not been fully elucidated. It appears that the therapeutic actions of lithium involve several complex and possibly inter-related processes (Malhi et al., 2013). Unlike other psychotropics, lithium does not bind to cellular receptors, but is known to interact with many elements of second messenger systems via enzyme inhibition, thereby modulating processes upstream and downstream of its targets and modulating neurotransmission (Stahl, 2008). Lithium appears to have neuroprotective properties and increases the volume of brain structures involved in emotional regulation (Bearden et al., 2007; Monkul et al. 2007; Hajek et al., 2013).

At a neuronal level it reduces excitatory, but increases inhibitory neurotransmission. It reduces oxidative stress caused by multiple episodes of mania and depression and increases the levels of protective proteins, such as brain-derived neurotrophic factor, and reduces apoptotic processes, thereby exerting some therapeutic action. Hypotheses regarding the mechanism of action of lithium include competition with magnesium, changes in expression of genes involved in inositol turnover and the inhibition of glycogen synthase kinase 3.

2.2.1. Competition with magnesium

A leading hypothesis to explain its mood-stabilising effect is that lithium competes with magnesium (Mg^{2+}) for metal-binding sites in enzymes that are involved in neurotransmitter pathways, but not in other vital Mg^{2+} -containing proteins. As Mg^{2+} is a co-factor of multiple enzymes this may explain several of lithium's biochemical effects (Amari et al., 1999; Ryves and Harwood, 2001).

The biochemical properties of lithium are closer to Mg^{2+} than to any other alkali metals in group IA of the periodic table of elements. Both lithium and Mg^{2+} are non-polarised cations, have high charge density and a strong affinity for "hard" O-containing ligands, as well as having similar ionic radii (Shannon, 1976). For lithium treatment to be effective, yet not life-threatening, it needs to compete with Mg^{2+} in some, yet not in other critical proteins. Mg^{2+} is favoured above lithium in most proteins by a solvent-inaccessible Mg^{2+} -binding site that is lined with negative charges e.g. Asp/Glu. There are, however, exceptions that allow the binding of lithium. Dudev and Lim (2011) have demonstrated that enzymes that are lithium-sensitive have Mg^{2+} -binding sites with a high positive charge density, some bulky ligands and high solvent exposure for dicationic, but not for tricationic complexes (Dudev and Lim, 2011).

In vitro, lithium can successfully compete with Mg^{2+} and bind to metal-containing sites in G-proteins (Srinivasan et al., 2004), GSK3 (Ryves and Harwood, 2001), IMPase (Leech et al., 1993), inositol polyphosphate phosphatase (Inhorn and Majerus, 1987), fructose-1,6-bisphosphate (Villeret et al., 1995) and the human erythrocyte membrane (De Freitas et al., 1994).

2.2.2. Regulation of Ion Exchange

The dysregulation of ion homeostasis has been reported to correlate with changes in the mood of bipolar patients, and some evidence points to lithium correcting this imbalance (Dubovsky et al., 1992; Herman et al., 2007).

All tissues in the body maintain a sodium electrochemical gradient that is required for a wide range of transport functions, e.g. glucose and amino acid uptake, ion transport and others. It is further essential to maintain cell excitability, to control intracellular sodium and calcium concentrations and intracellular pH (El-Mallakh and Jaziri, 1990; Fiekers, 2001).

The symptoms of BPD can partly be explained by high intracellular sodium and calcium levels in the brain, as these result in an increase in neurotransmitter release and reduce the membrane potential to increase cell excitability. The intracellular sodium concentrations of depressed or manic BPD patients have been reported to be two to five times that of normal levels (Shaw, 1966; Naylor et al., 1970). It has been observed that lymphoblasts and lymphocytes of BPD patients have a lower capacity to respond to increased intracellular sodium levels (Wood et al., 1991), which is reported to involve dysregulation of $\text{Na}^+\text{-K}^+\text{-ATPase}$ expression (El-Mallakh and Wyatt, 1995). A reduction in the activity of this pump has been observed in patients, particularly when they are in the depressed phase, which is associated with an increase in sodium retention (El-Mallakh et al., 1996). A pathway-based analysis of GWAS studies in BPD has also revealed that the most enriched gene sets associated with this phenotype are ion channel structural and regulatory genes (Askland et al., 2009).

Lithium, as well as other mood stabilisers, such as CBZ, VPA and LTG, reduces intracellular sodium and calcium concentrations, thereby non-specifically inhibiting neurotransmitter release (Farber et al., 2002; Hahn et al., 2004; Wasserman et al., 2004). Lithium decreases intracellular sodium concentrations, through two different mechanisms. Lithium can displace sodium on a one to one basis as it accumulates within electrically active tissues (Haas et al., 1975), causing a decrease in 24 hour exchangeable sodium and residual sodium in lithium-treated patients (Coppen et al., 1965). Long-term lithium treatment also causes an increase in $\text{Na}^+\text{-K}^+\text{-ATPase}$ activity in erythrocyte membranes, with concurrent reduction in sodium and calcium in the erythrocytes of BPD patients (Hokin-Neaverson and Jefferson, 1989). Additionally, lithium reduces N-methyl-D-aspartate (NMDA) receptor mediated Ca^{2+} -influx (Chuang, 2004; Ghasemi et al., 2010), further normalising intracellular calcium levels in BPD patients (Dubovsky et al., 1992). Lithium's influence on sodium and calcium levels within the brain may play a role in the mood stabilising action of lithium in patients with BPD.

2.2.3. Lithium's effect on Gene Expression and Activity

Lithium is known to disrupt the activity of enzymes involved in inositol turnover and inhibits Glycogen synthase kinase 3 (*GSK3*), which suggests the potential involvement of the inositol and Wnt signalling pathways in lithium action (Berridge et al., 1989; Hedgepeth et al., 1997). Other signalling pathways, such as cyclin dependent kinase-5, protein phosphatase 1, protein phosphatase 2A and MEK/ERK have also been found to be regulated by lithium (Mora et al., 2002; Pardo et al., 2003; Jordā et al., 2005). In rodent brains, lithium treatment induces several changes in gene expression and is therefore thought to be involved in many cellular processes, although the exact neuronal processes are not completely understood (Fatemi et al., 2009). The main candidates to explain lithium's mechanism of action are *GSK3* and inositol-related genes.

2.2.3.1. *Inositol phosphate depletion*

The enzyme inositol monophosphatase (IMPase) was the first identified target of lithium (Hallcher and Sherman, 1980). IMPase is involved in the formation of phosphatidyl inositol (PI) through the dephosphorylation of inositol monophosphate to release myo-inositol. Inositol monophosphatase is formed from inositol phosphate (IP3) through two dephosphorylation steps, the latter completed by inositol 1-polyphosphate phosphatase (IPP), a second lithium sensitive enzyme (Inhorn and Majerus, 1988).

Due to lithium's action on two enzymes involved in its formation, the synthesis of myo-inositol is sensitive to lithium. Intra-cellular levels of inositol can therefore be lowered by lithium treatment (Harwood, 2004). In cells, inositol depletion can cause a significant change in inositol phosphate signalling and lithium may potentially enhance the effect, through additionally lowering inositol up-take (Calker and Belmaker, 2000).

Gene expression studies on human neuronal cell lines, have identified several genes associated with inositol metabolism to be up- or down-regulated, following chronic lithium treatment (Seelan et al., 2008). Levels of IP3, a downstream product of inositol metabolism, are lowered by lithium. IP3 is a second messenger that binds to the endoplasmic reticulum releasing calcium (Ca^{2+}) from its intracellular store (Mikoshiba et al., 1993). The transient elevation of Ca^{2+} affects several cellular processes, including cell motility, cell survival and neurotransmitter release.

Behavioural changes due to inhibition of inositol phosphatases by lithium have been reported in the social amoeba, *Dictyostelium* (King et al., 2009) and in *Caenorhabditis elegans* (Tanizawa et al., 2006). In mammals, however, inositol depletion does not mimic lithium's effects on behaviour. In mice, for example, a heterozygous deletion of the *sodium dependent myo-inositol cotransporter-1 (SMIT-1)* reduces myo-inositol levels to the same degree as lithium, but has no effect on behavioural phenotypes affected by lithium (Shaldubina et al., 2006).

2.2.3.2. Glycogen synthase kinase 3

The GSK3 serine threonine kinases family, composed of two isoenzymes GSK3A and GSK3B, have several biological roles including implications in the regulation of development, immunity/inflammation, cancer and several aspects of neuronal function (Cohen and Frame, 2001; Woodgett, 2001; Kaidanovich-Beilin and Woodgett, 2011). Dysregulation of GSK3 has understandably been associated with a number of clinical conditions, such as diabetes, oncogenesis and neurodegeneration.

Lithium inhibits GSK3, both directly and indirectly. Lithium firstly acts as a non-competitive inhibitor of the binding of Mg^{2+} , a co-factor of GSK3 (Ryves and Harwood, 2001); and further affects GSK3 by activation of the serine/threonine kinase, AKT, resulting in an increase of GSK3 phosphorylation on the Ser 9 residue and consequent inactivation in cultured neurons (Chalecka-Franaszek and Chuang, 1999). In rodents, the inhibition of GSK3 activity could mimic both the "antidepressant" and "antimanic" effects of lithium (Beaulieu et al., 2004). GSK3 has several substrates and its inhibition by lithium, therefore has a resultant effect on many different pathways with myriad downstream effects. The regulation of GSK3 by lithium and its effect on gene expression, synaptic plasticity, and neurogenesis, which in turn regulate mood-related behaviours, is demonstrated in Figure 2.1 (Li and Jope, 2010).

Lithium's inhibition of GSK3 can mediate its mood-stabilising effect through upregulation of cyclic AMP (cAMP) response element-binding protein (CREB) (Mai et al., 2002), the over-expression of which has an antidepressant-like effect in the dentate gyrus (Chen et al., 2001). GSK3B has been implicated in the regulation of the direction and magnitude of N-methyl-D-aspartate receptor (NMDAR) dependent plasticity at excitatory synapses (Peineau et al., 2007; Hooper et al., 2007). GSK3B further regulates a large group of transcription factors and transcriptional modulators and could therefore be responsible for the regulation of the expression of genes, involved in mood disorders and mood regulation (Shaltiel et al., 2007).

In addition to its role in the activation of neuroprotective neurotrophins, such as brain-derived neurotrophic factor (BDNF), the inhibition of GSK3 is known to have intracellular neuroprotective actions. GSK3 inhibitors can reduce many types of apoptosis-inducing insults, such as misfolded protein accumulation and DNA damage (Song et al., 2002; Watcharasit et al., 2002).

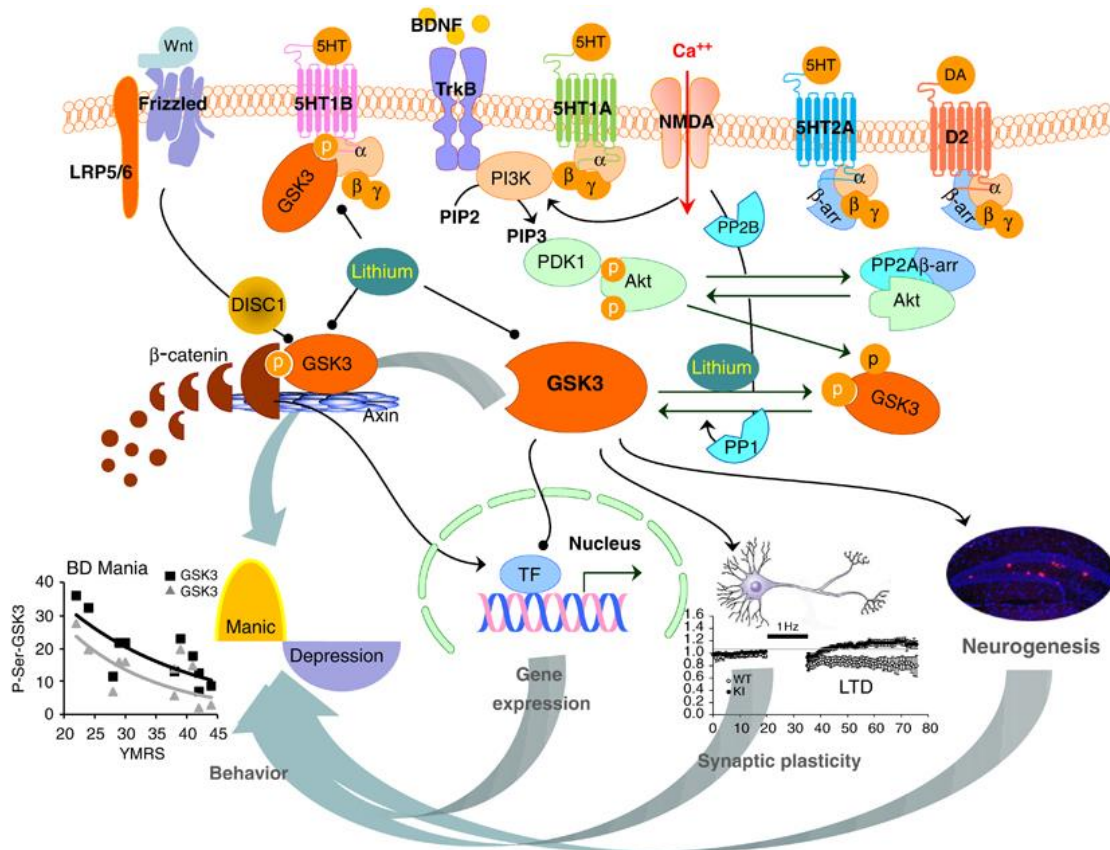


Figure 2.1: Illustration of regulation of GSK3 by lithium via signalling pathways and functions of GSK3 inhibition in mood regulation. GSK3 is regulated by BDNF, serotonin, and dopamine through the Akt signalling pathway, Wnt signalling and by the NMDA receptor through protein phosphatases. GSK3 affects gene expression, synaptic plasticity, and neurogenesis, which in turn regulate mood-related behaviours. Abbreviations: α,β, γ: G-protein subunits; β-arrest: β-arrestin; BD: bipolar disorder; BDNF: brain-derived neurotrophic factor; DA: dopamine; D2: type 2 dopamine receptor; DISC1: disrupted in schizophrenia 1; GSK3: glycogen synthase kinase-3; 5HT: serotonin; 5HT1A, 1B, 2A: serotonin receptor subtypes; LTD: long term depression; NMDA: N-methyl-D-aspartic acid; P: phosphorylated; PDK1: phosphoinositide-dependent kinase-1; PI3K: phosphatidylinositol-3-kinase; PP1, PP2A, PP2B: protein phosphatase 1, 2A, and 2B; TF: transcription factors; TrkB: type B tropomyosin-receptor-kinase; YMRS: Young Mania Rating Scale (Image from Li and Jope, 2010).

Neuroprotection helps cells to respond better to stress, as well as enhancing neuronal functions that could counteract some stress-induced mood changes. GSK3 inhibits the transcription factor HSF-1, which causes a reduction in the expression of chaperone proteins (Chu et al., 1996). Chaperone proteins provide protection against neuronal insults, and the inhibition of GSK3 by lithium leads to an upregulation in expression of these chaperones, thereby strengthening cellular responses to stress. GSK3 also promotes impairments associated with DNA damage, such as in the nonhomologous end-joining DNA repair pathway, which is promoted by GSK3 inhibition (Yang et al., 2009).

GSK3 plays a major role in regulating the innate and adaptive immune responses, which has an important influence on particularly the pathology and treatment of depression (Miller et al., 2009). GSK3 inhibition by lithium leads to a reduction of several pro-inflammatory cytokines and, conversely, an increase in the levels of the anti-inflammatory cytokine, IL-10, which was demonstrated to rescue approximately 70% of investigated mice from an otherwise lethal inflammatory response to lipopolysaccharides (Martin et al., 2005).

Lithium is also able to slow the abnormally fast circadian rhythms found in many bipolar patients (Welsh and Moore-Ede, 1990; Klemfuss, 1992; Hafen and Wollnik, 1994; Abe et al., 2000; Dokucu et al., 2005), which is possibly mediated by *GSK3B*. *GSK3B* can modify several clock genes and has been implicated in the regulation of the stability and/or nuclear translocation of *PER2*, *CRY2*, *CLOCK*, *REV-ERBa* and *BMAL1* (Harada et al., 2005; Yin et al., 2006; Spengler et al., 2009; Ko et al., 2010; Sahar et al., 2010).

In addition, GSK3 is an essential element of the Wnt/beta-catenin pathway critical for patterning, cell fate specification, and stem cell regulation during the development of many tissues and organs, including the mammalian brain (reviewed by Kim and Snider, 2011). In adults, Wnt signalling regulates stem cell homeostasis and cell proliferation, thereby continuing to influence the maintenance and regeneration of many tissues (reviewed by Wend et al., 2010). In the brain, Wnt signalling is especially important as it regulates neurite outgrowth, axon remodelling, synapse formation and plasticity, and neurogenesis – processes critical for normal brain function (Budnik and Salinas, 2011).

2.2.4. Pharmacogenetic studies on lithium response

Pharmacogenetic studies on lithium response have focused on the pharmacodynamic aspects of the drug, due to the lack of empirical evidence for a relationship between plasma drug levels and lithium efficacy. Linkage studies, candidate gene studies, GWAS and gene expression studies have been performed to identify genetic determinants of lithium treatment response, thorough reviews of which have been conducted elsewhere (McCarthy et al., 2010; Smith et al., 2010; Rybakowski et al., 2013a)

Several candidate gene studies on lithium response have been performed on inositol-related genes, such as *PLCG1*, a gene coding for phospholipase $\text{C}\alpha_1$, a key enzyme involved in the inositol pathway, as well as G protein-mediated signals. An association has been reported for a dinucleotide repeat in *PLCG1* with excellent response to lithium (Turecki et al., 1998), although these findings were not replicated in a sample of Norwegian lithium-treated bipolar patients (Løvlie et al., 2001).

Studies investigating other elements of the inositol pathway have reported association for the silent variant C937A in the gene inositol polyphosphate 1-phosphatase (*INPP1*) and response to lithium (Steeir et al., 1998), but this association was not replicated in other studies (Michelon et al., 2006). No association with lithium response has been reported for variants in inositol monophosphatase 1 (*IMPA1*) (Calabrese et al., 1996).

Lithium is known to regulate the expression of neurotransmitters and elements of these systems, such as the BDNF/TrkB signalling pathway which has been studied for its involvement in lithium response and BPD. An association has been reported for SNP rs6265 (Val66Met) in the *BDNF* gene and better response to lithium (Rybakowski et al., 2007). A recent study has additionally shown lithium treatment to be associated with a significant reduction of DNA methylation in the promoter region of the *BDNF* gene when compared to other drugs (D'Addario et al., 2012). An association with a polymorphism in the Src-family tyrosine kinases (*FYN*) gene and lithium response has also been reported. *FYN* belongs to a family of protein kinases that phosphorylates the NMDA receptor subunits that play a role in regulating the BDNF/TrkB signal transduction pathway (Szczepankiewicz et al., 2009).

Lithium is known to upregulate cAMP response element-binding protein (CREB) (Mai et al., 2002) and genetic variation in genes coding for CREB proteins have therefore been investigated for a role in determining response to lithium prophylaxis (Mamdani et al., 2008). Mamdani and colleagues (2008) reported the SNPs rs6740584 and rs2551640 in *CREB1* to be associated with response to lithium.

Serotonin- and dopamine-related genes have also been investigated; associations with lithium response and variants in the serotonin transporter gene (5'-*HTTLPR*), tryptophan hydroxylase (*TPH*) and dopamine receptor D1 (*DRD1*) have been found (Serretti et al., 1999; Rybakowski et al., 2005; Rybakowski et al., 2009).

Only one GWAS study for lithium response in bipolar patients has been published to date and no SNP met the threshold for genome-wide association (Perlis et al., 2009). However, five regions showed consistent evidence of association in a second cohort of patients treated with lithium (Perlis et al., 2009). One of these regions includes the gene coding for a subunit of the ionotropic glutamate receptor gene, *GLUR2/GLURB*, which binds to alpha-amino-3-hydroxy-5-methyl-4-isoxazolpropionate (*AMPA*). This gene, *GRIA2* (glutamate/AMPA receptor), is also downregulated by chronic lithium treatment in human neuronal cell lines (Seelan et al., 2008).

In 2008, the Consortium on Lithium Genetics (ConLiGen, www.conligen.org) was created. This is an international joint effort, with sample size currently comprising of more than a 1200 patients, characterised for response to lithium using a stringent phenotype definition (Schulze et al., 2010). The scale used to determine phenotype quantifies the degree of improvement over the duration of treatment with a score from 0 to 10, where patients with a total score of seven or above are considered full responders (Manchia et al., 2013). ConLiGen aims to assess all aspects of lithium pharmacogenetics, including genetic susceptibility to potential adverse effects due to treatment. The first objective of the project will be to conduct a GWAS of response to lithium treatment in bipolar disorder. Preliminary reports from this study suggest the possible involvement of *SLC4A10*, the sodium lithium bicarbonate transporter gene, in lithium response (Rybakowski, 2013a).

Chapter 3. Lithium-induced Adverse Drug Reactions

It is evident that lithium influences multiple pathways that are often interconnected and can have an infinite number of effects on neuronal as well as other systems within the human body. The current disease model of BPD is that it is a complex polygenic disorder with high heterogeneity. Not all patients will, therefore, have the exact same underlying combination of genes or pathways involved in their disease. When a drug such as lithium acts on multiple systems, it will have off-target effects leading to ADRs.

Non-adherence to lithium treatment, the main cause of which is ADRs, is one of the major factors that lower its effectiveness in clinical practice (Maj et al., 1998; Schumann et al., 1999). Approximately 75%-90% of patients on long-term lithium treatment experience one or more ADRs (Dunner, 2000). The multiple ADRs of lithium use include thyroid dysfunction, neurological, gastrointestinal, metabolic, dermatological, nephrogenic, cognitive and sexual side effects (reviewed by Dols et al., 2013). Combating these side effects could therefore be an important factor in improving the persistence on lithium treatment. Attempts at pharmacogenetically determining factors associated with the development of one or more of these ADRs, could provide clues for ways of effectively treating the ADRs, as well as improving our understanding of mechanisms of action of this drug.

Due to interindividual differences in PGx, lithium dose is not a good predictor of its effectiveness or the risk of developing ADRs. There is, however, a positive correlation between lithium serum levels and the occurrence and severity of ADRs (Wilting et al., 2009). The monitoring of lithium serum levels is, therefore, currently not only critical to prevent the development of lithium toxicity, but also to prevent ADRs. Lithium serum levels are advised to remain at 0.6-0.8mmol/L for maintenance treatment and at 0.8-1.2mmol/L for the treatment of acute manic episodes (Amdisen, 1977).

To date, no studies have been conducted on the prevalence of lithium ADRs in a South African population. There is, however, some evidence that there may be inter-population differences in the frequency of ADR occurrence. For example, African Americans have been reported to have a higher prevalence of ADRs than their Caucasian counterparts in the United States, even when lithium is within the therapeutic range (Strickland et al., 1995). This may be due to the higher red blood cell to plasma ratio of lithium in African Americans when compared to Caucasians (Strickland et al., 1995) or Asians (Okpaku et al., 1980).

3.1. Nephrogenic side effects

Two of the most common ADRs in lithium-treated patients are polydipsia (increased thirst) and polyuria (increased urinary volume), which are largely due to lithium's effect within the kidneys. Patients on long term lithium treatment often have an irreversible reduction in maximal urinary concentrating ability, which could lead to nephrogenic diabetes insipidus (NDI). NDI is characterized by increased water and sodium diuresis, resulting in mild dehydration, hyperchloremic metabolic acidosis and renal tubular acidosis. There is a risk of developing hypernatraemic dehydration if the patient is unable to drink, which is a particular risk factor in the elderly (Mukhopadhyay et al., 2001). The risk of developing NDI correlates with the duration of lithium therapy and this may occur as early as 8 weeks after treatment initiation and be present in 10-40% of patients (Bendz and Aurell, 1999). In patients treated long term, NDI is only partly reversible when lithium treatment is stopped, suggesting some permanent renal damage.

In the kidney, lithium is freely filtered through the glomeruli and nearly 80% is reabsorbed in the renal proximal tubule and a small fraction in the distal parts of the nephron through the epithelial sodium channel (ENaC). The ENaC is permeable only to lithium and sodium, with its permeability to lithium being 1.5-2 fold higher than for sodium (Grünfeld and Rossier, 2009). Sodium is exported from the interior of the cells into the blood by the sodium-potassium-transporting ATPase (Na^+/K^+ -ATPase) pump, for which lithium is a poor substrate (Grünfeld and Rossier, 2009). Lithium-induced NDI can be treated with amiloride, a blocker of ENaC (Grünfeld and Rossier, 2009). The transport of sodium, potassium and water in a cell of the cortical collecting duct of the kidney under basal conditions compared to in the presence of lithium is demonstrated in Figure 3.1.

Lithium can accumulate intracellularly in the collecting duct where the inhibition of the GSK3B enzyme by lithium (section 2.2.3.2) leads to dysregulation in water and sodium transport. Subsequently, the cell becomes insensitive to aldosterone and vasopressin actions, resulting in water and sodium diuresis (Decloedt and Maartens, 2011). Lithium also causes dysregulation in the expression and trafficking of the aquaporin 2 (AQP2) water channel along the entire collecting duct, which might be related to a decrease in interstitial osmolarity. Mutations in AQP2 causes inherited NDI and this protein therefore has an important role in renal physiology. Cellular levels of AQP2 were observed to be reduced by lithium (Oksche and Rosenthal, 1998) through the reduction of AQP2 mRNA levels (Laursen et al., 2004).

AQP2 down-regulation in rat collecting ducts was observed after only four weeks of lithium treatment, which was only partly reversed with withdrawal of lithium (Mønster et al., 2004). The downregulation of AQP2 is influenced by the inhibition of *GSK3B* and the subsequent inhibition in cAMP recruitment of AQP2. A study of the inner medullary collecting ducts of lithium-treated rats demonstrated that components of several signalling pathways, including protein kinase B (AKT) (section 3.7.2) and mitogen- activated protein kinases, are activated by lithium treatment in the kidney (Li et al., 2006).

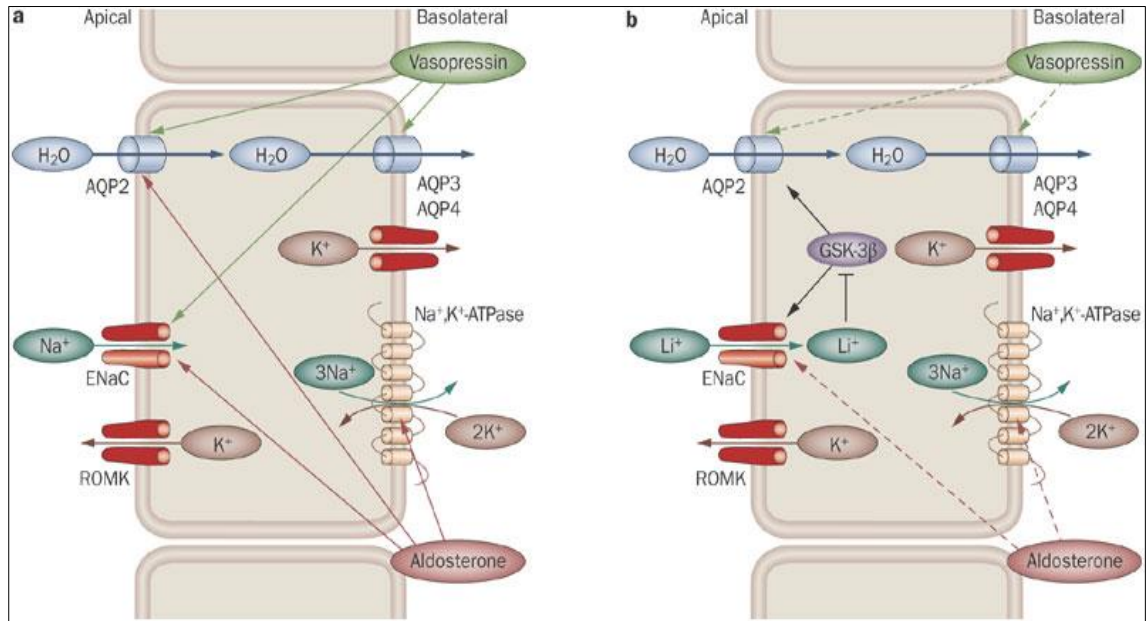


Figure 3.1: The transport of sodium, potassium and water in a cell of the cortical collecting duct of the kidney under basal conditions compared to in the presence of lithium. a) Under basal conditions sodium enters the cell through ENaC along an electrochemical gradient and it is actively transported by the Na⁺-K⁺-ATPase pump. Potassium enters the cell through Na⁺-K⁺-ATPase and is secreted through rOMK, along the electrochemical gradient created by the entry of sodium. Water enters the cell through AQP2 and exits through AQP3 and AQP4. The transport of sodium and water is controlled by the hormones, aldosterone and vasopressin. b) During lithium treatment, lithium enters the cell through ENaC, preventing the entry of sodium. As the Na⁺-K⁺-ATPase pump is impermeable to lithium, lithium accumulates in the cell and inhibits GSK3B, which affects the levels of AQP2 and ENaC. The cell becomes partially insensitive to aldosterone and vasopressin action and lithium therefore leads to water and sodium dysregulation in the kidneys (Image from Grünfeld and Rossier, 2009).

A more concerning side effect of long-term lithium treatment is chronic tubulointerstitial nephropathy, which is characterised by decreased glomerular filtration rate and chronic kidney disease. Interstitial fibrosis can present as early as five years after treatment initiation and may develop chronically in the absence of episodes of lithium intoxication (Presne et al., 2003), and can further lead to the development of end-stage renal disease. In a recent meta-analysis the absolute risk of developing renal failure was only 0.5% (McKnight et al., 2012).

Lithium-induced nephropathy can be detected by testing for elevated serum creatinine measurements, but patients that are efficiently managed with lithium are often not regularly followed-up, and renal failure is, therefore, often detected too late. The practice guidelines of the American Psychiatric Association recommend measurement of serum creatinine level every 2–3 months during the first 6 months of lithium therapy and every year thereafter (Alexander et al., 2007). Patients in rural areas are often not treated with lithium at all, in spite of it being the first-line treatment in BPD, due to fears of them developing renal failure without access to clinics for regular monitoring of creatinine levels. This is particularly pertinent to settings such as in South Africa and the rest of the African continent.

3.2. Dermatological side effects

Lithium-induced dermatological side effects were first described in the late 1960's (Callaway et al., 1968). Various skin problems associated with lithium use, including psoriasis, acneiform eruption, exfoliative dermatitis, pityriasis, versicolour, pruritic maculopapular erythematous eruption, dermatitis herpetiformis and Darier's disease have since been reported (Heng, 1982; Jenni and Krebs, 1984; Rubin, 1995). The reported prevalence of these dermatological side effects vary from 3.4 to 45% among lithium-treated patients (Jafferany, 2008), with the most common symptoms being acne vulgaris and psoriasis (Chan et al., 2000). In contrast with acute lithium toxicity, most dermatological side effects occur at therapeutic serum lithium levels and it can have a dramatic effect on patient compliance (Chan et al., 2000).

The mechanism by which lithium causes various dermatological side effects is unknown, although these side effects are commonly associated with pathologic findings of neutrophilic infiltration in the epidermis. Lithium's effect on G-proteins, its inhibition of adenyl cyclase and inositol monophosphatase (leading to lower cAMP and inositol levels), coupled with the inhibition of *GSK3* have been proposed to lead to neutrophilia, and the consequent potential development of dermatoses (Jafferany, 2008).

Decreased cAMP has been proposed to lead to keratinocyte proliferation, increased chemotaxis and phagocytic activity. These factors are all commonly involved in the pathogenesis of acneiform eruptions, psoriasiform eruption, pustular psoriasis, and other conditions characterized by neutrophilic infiltration. Lithium can cause dermatological diseases both *de novo* and through the exacerbation of pre-existing conditions.

Psoriasis can possibly be caused or exacerbated by the decreased inositol levels in the skin caused by lithium. Inositol depletion leads to the inhibition of calcium release and calcium levels are important in the process of keratinocyte differentiation and proliferation. Psoriatic keratinocytes do not undergo complete differentiation and lack the elevated intracellular calcium levels necessary for terminal differentiation (Hwang et al., 2001). In support of this theory, Grisaru and Belmaker (1994) published a report of a patient with lithium-exacerbated psoriasis that was dramatically improved by peripheral inositol supplementation (Grisaru and Belmaker, 1994). Once the daily dose of inositol was removed, the psoriasis worsened, but improved again upon the reapplication of inositol. Peripheral inositol is available in peripheral tissue, such as the skin, but does not cross the blood-brain barrier and, therefore, do not alter lithium's effect on the brain (Allan et al., 2004). Similarly, a double-blind, randomized, placebo-controlled, crossover trial found a significant improvement in the psoriasis of patients on lithium receiving an inositol supplement compared to patients receiving a placebo (Allan et al., 2004).

3.3. Metabolic effects

Weight gain is common among patients with BPD, with overweight and obesity ranging from 20% to 35%, exceeding the prevalence in the general population (McElroy et al., 2002; Fagiolini et al., 2002; McElroy et al., 2004). At a Bipolar Disorder centre in Pennsylvania, 49% of patients had abdominal obesity and 30% displayed metabolic syndrome (Fagiolini et al., 2005). Metabolic syndrome is especially dangerous as it is associated with other clinical conditions, including insulin resistance, dyslipidaemia, central obesity, hypertension, impaired glucose tolerance and high rates of atherosclerotic disease (Toalson et al., 2004). Weight gain is an adverse effect of several psychiatric drugs used in the treatment of BPD. In addition to medication, weight gain in BPD patients can also be influenced by atypical depression, comorbid eating disorders or merely disturbed eating habits (Torrent et al., 2004; Wildes et al., 2006).

Treatment-induced weight gain can occur in a short period after treatment commencement and patients therefore develop a negative view of the treatment, which often negatively impacts treatment compliance – even in patients experiencing an amelioration of their affective symptoms. In patients on lithium prophylaxis, weight gain was related to poor compliance in up to 50% of cases, and 90% of cases considered treatment discontinuation (Goodwin and Jamison, 2007).

Weight gain is reported to be present in 25% of patients on lithium treatment (Goodwin and Jamison, 2007) and one study has suggested that over 30% of lithium-treated patients are obese (Silverstone and Romans, 1996). Weight gain has been found to increase in the first 1-2 years of treatment and then remain constant (Vestergaard et al., 1988). It can be explained by fluid retention, increased appetite, lithium-related subclinical hypothyroidism or a combination of these factors (reviewed by Torrent et al., 2008). In addition, lithium may affect carbohydrate metabolism, through insulin-like activity, leading to increased glucose absorption in adipose tissue (Shopsin et al., 1972; Müller-Oerlinghausen et al., 1979). Another potential factor is polydipsia (increased thirst), which can cause some patients to drink large quantities of high-caloric drinks, as shown in a study of weight gain in patients with BPD (Elmslie et al., 2001). Lastly, it has been reported that lithium may have a direct effect on the hypothalamus to stimulate appetite and thirst (Kulkarni and Kaur, 2001).

3.4. Endocrine side effects

Lithium is associated with the development of hypothyroidism by affecting the thyroid gland function. The prevalence of hypothyroidism is as high as 47% in patients taking lithium, with females being five times more at risk than males (Esposito et al., 1997; Henry, 2002). The lithium ion is concentrated in the thyroid gland where it interferes with the production of thyroid hormones (TH). Lithium inhibits iodine uptake, as well as altering thyroglobulin structure. TH secretion is also inhibited by lithium, possibly through its interference with tubulin polymerisation (Burrow et al., 1971; Bhattacharyya and Wolff, 1976). Subclinical hypothyroidism is present in 8% of females and 3% of the general male population, and these patients are at increased risk for developing hypothyroidism on lithium.

Lithium-induced reduction in TH release, results in a reduction in feedback inhibition upon the pituitary and consequent increase in thyroid stimulating hormone (TSH) secretion (Schiemann and Hengst, 2002). A goitre (swelling of the thyroid gland) can develop within weeks or only after several years of lithium treatment and occurs in 5% to 55% of patients treated with lithium (Kibirige et al., 2013).

The large variation in reported frequency can be explained by differences in iodine content in the geographical study settings, the duration of lithium use and different study designs. In addition, lithium is known to induce a spectrum of calcium homeostasis disorders, including cases of overt hyperparathyroidism (HPT) and cases of hypercalcemia without elevated parathyroid hormone (PTH) levels or elevated PTH without hypercalcemia (Nordenstrom et al., 1994; Komatsu et al., 1995). The prevalence of lithium-associated HPT is estimated to range from 4.3% (Awad et al., 2003) to 6.3% (Bendz et al., 1996), which is higher than the overall prevalence of HPT in the general population (1–4/1000) (Melton, 1991).

3.5. Neurological side effects

Tremor is a frequent side effect of lithium and occurs in 4% to 65% of patients, with a recent review estimating the average percentage at 27% (Gelenberg and Jefferson, 1995). Tremor has an influence on patient compliance and in one study 32% of patients reported lithium tremor to result in some disability and non-compliance (Morgan and Sethi, 2005). Severe tremor can be one of the first signs of lithium toxicity.

The pathophysiology of lithium tremor has, surprisingly, never been studied (Baek et al., 2013), but it is thought to arise from the central nervous system (Elble, 1996; Deuschl et al., 2011) as lithium affects neural plasticity (Soeiro-de-Souza et al., 2012). Physiological tremor is reported to result from a central oscillator, originating in the inferior olive (Lamarre, 1979) or thalamus (Semba et al., 1980) and transmitted through the corticospinal tract (Köster et al., 1998).

Lithium tremor is thought to be influenced by changes in brainstem serotonergic neurons, due to the negative synergistic effect in patients that are treated with other pro-serotonergic agents, such as antidepressants (Elble, 1996). Lithium's effects on grey matter volume (Monkul et al., 2007) and the microstructure of white matter (Benedetti et al., 2012) may additionally affect the corticospinal tract and central oscillator transmission.

3.6. Other side effects

Gastrointestinal side effects, such as nausea, vomiting, and diarrhoea are common, although generally transient, side effects of lithium treatment (Bowden et al., 2000), occurring in up to 50% of patients. These side effects can be symptoms of lithium toxicity, but are often due to excessive dosage levels in the lithium-naïve (Macritchie and Young, 2004).

Poorer cognitive performance is a common impairment in BPD patients, independent of treatment (Martinez-Aran et al., 2005; Young et al., 2006). Lithium therapy has been associated with poorer performance on tests of memory and motor speed (Pachet and Wisniewski, 2003), although these negative effects on cognition appears to be minor (Wingo et al., 2009).

3.7. Candidate genes

Although not previously investigated, we propose that lithium pharmacogenetics may play a role in interindividual susceptibility to lithium-related ADRs. Determining genetic factors associated with susceptibility to side effects, may aid in optimising prescription of lithium in Bipolar Disorder, as other first or second-line treatments could be prescribed for individuals with a high susceptibility to serious lithium-related side effects, or these could be monitored more carefully. Table 3.1 gives a summary of candidate genes and SNPs in these genes that will be investigated.

3.7.1. Glycogen Synthase Kinase 3 Beta (*GSK3B*)

The first candidate gene to be investigated for its potential role in lithium-induced side effects, *GSK3B*, has been discussed in detail in section 2.2.3.2.

Three SNPs in *GSK3B* will be genotyped in the patient cohort. The first SNP, rs334558, is located in the promoter region and influences expression of the gene as the T allele has a greater transcriptional strength than the C allele (Kwok et al., 2005). This variant has been associated with response to lithium (Benedetti et al., 2005; Lin et al., 2012). Lithium-treated patients homozygous for the C allele of this variant have also been reported to have better renal concentrating ability than carriers of the T allele, and may therefore be more resilient to lithium *GSK3B* inhibition in the kidneys (Rybakowski et al., 2013b). The SNP, rs3755557, is in the promoter region and functional assays have demonstrated it to influence the binding affinities of transcription factors, resulting in the risk allele having a higher promoter activity (Kwok et al., 2005; Li et al., 2011). The third SNP, rs6438552, located in intron 5, influences *GSK3B* alternative splicing. *GSK3B* has two alternatively spliced exons (9 and 11) that are predicted to generate four different splice isoforms (Schaffer et al., 2003). The T allele of rs6438552 results in greater levels of the *GSK3B* exon9+11 isoform and increased enzyme activity in lymphocytes (Kwok et al., 2005).

Table 3.1: Candidate genes and respective SNPs to be investigated for association with lithium-induced side effects

Gene	Chromosomal Location	NCBI RefSNP ID	HGVS name	SNP location	Protein
GSK3B	3q13	rs334558	NM_001146156.1: c.-983-18T>C	Promoter	
		rs3755557	NM_001146156.1: c.-983-1693A>T	Promoter	
		rs6438552	NM_001146156.1: c.609-157T>C	Intron 5	
AKT1	14q32	rs10138227	NM_005163.2: c.-322G>A	Promoter	
		rs1130233	NM_005163.2: c.726G>A	Exon 9	Glu242=
ARRB2	17p13	rs34230287	NM_004313.3: c.-228-159C>T	Promoter	
		rs1045280	NM_004313.3: c.840C>T	Exon 11	Ser280=
GRIA2	4q32	rs4302506	NM_000826.3: c.687T>C	Exon 5	His229=
PPARGC1a	4p15	rs2279525	NM_013261.3: c.*3193A>G	5'UTR	
		rs8192678	NM_013261.3: c.1444G>A	Exon 8	Gly482Ser

HGVS: Human Genome Variation Society

3.7.2. V-akt murine thymoma viral oncogene homolog 1 (AKT1) and β -arrestin 2 (ARRB2)

AKT1 is one of three closely related serine/threonine-protein kinases (AKT1, AKT2 and AKT3) called the AKT kinase, also known as Protein Kinase B. Activation of Akt has been found in the striatum, frontal cortex, and hippocampus of rodents following either acute or chronic treatment with lithium (Beaulieu et al., 2004). AKT1 has an important function in cell growth, differentiation, plasticity, as well as several key neurodevelopmental pathways (Lai et al., 2006). Polymorphisms in *AKT1* have been associated with schizophrenia (Thiselton et al., 2008; Norton et al., 2007) and frontostriatal mediated cognitive task performance (Tan et al., 2008).

β -arrestin 2 (ARRB2) is one of the arrestin proteins that were first described for their role in desensitizing G-protein-coupled receptors (GPCRs). β -arrestin 2 is involved in cell communication and signal transduction and has previously been associated with schizophrenia (Liou et al., 2008). The *ARRB2* gene is down-regulated by lithium (Fatemi et al., 2009).

AKT and ARRB2, together with protein phosphatase 2A (PP2A) form a signalling complex which is dissociated in response to therapeutic levels of lithium treatment. This complex is formed following stimulation of the dopamine D2 receptor (DRD2) and its formation leads to the dephosphorylation and inactivation of AKT (Beaulieu et al., 2005). In mice lacking *Arrb2*, in which this signalling complex cannot be formed, lithium treatment failed to activate Akt and phosphorylate GSK3 (Beaulieu et al., 2008). A model of this inhibition, as described by Beaulieu and colleagues (2008) is demonstrated in Figure 3.2. Furthermore, lithium also failed to regulate GSK3 phosphorylation in mice with reduced Akt1 activity (Pan et al., 2011). Disruption of the complex by lithium leads to AKT activation, increase of GSK3 phosphorylation on the Ser9 residue and consequent inactivation of GSK3 in cultured neurons (Chalecka-Franaszek and Chuang, 1999).

The mechanism of lithium's interference with the formation of the Akt:ARRB2:PP2A signalling complex is not fully understood. However, *in vitro* experiments suggest that Mg^{2+} is required for the interaction of Akt and ARRB2 and, consequently, the formation of the complex, suggesting the role of lithium- Mg^{2+} competition (Beaulieu et al., 2008).

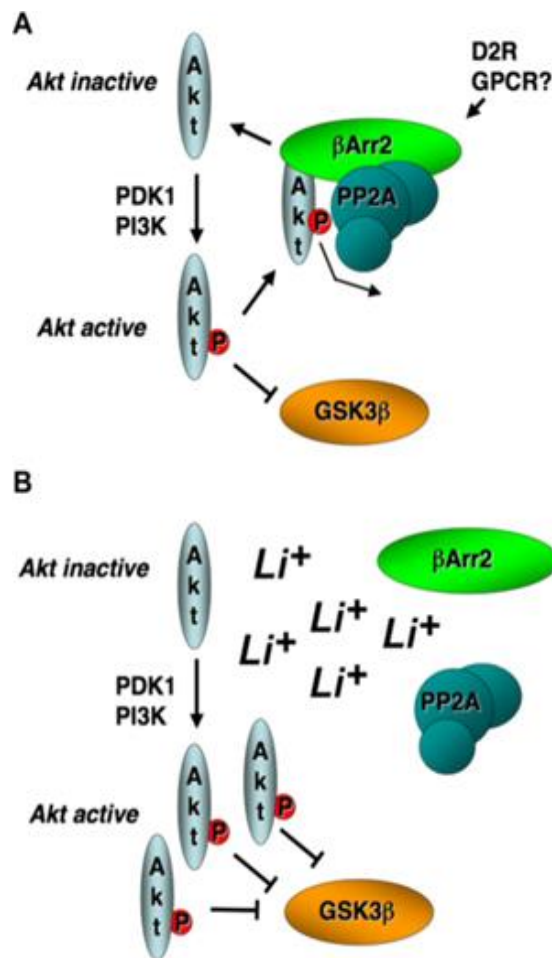


Figure 3.2: A model for the indirect inhibition of GSK3B activity.

(A) Under normal conditions, Akt is phosphorylated and activated leading to the phosphorylation and inhibition of GSK3B. Equilibrium is maintained between Akt phosphorylation/activation (by PI3K/PDK1) and its inactivation. Akt inactivation/dephosphorylation is facilitated by the formation of a signaling complex whereby bArr2 scaffolds Akt and PP2A in response to stimulation of GPCRs, such as the D2 DA receptor.

(B) Lithium destabilizes the signaling complex formed by Akt, bArr2, and PP2A, thereby enhancing Akt activity by reducing its dephosphorylation. In turn, increased Akt activity results in a greater inhibition of GSK3B.

(Figure adapted from Beaulieu et al., 2008)

Due to both the crucial role of this complex in lithium's inhibition of *GSK3*, as well as the important functions of these genes within the brain, polymorphisms in *AKT1* and *ARRB2* may lead to interindividual differences in patient response. The SNPs rs1130233 and rs10138227 in *AKT1* and rs1045280 and rs34230287 in *ARRB2* will be investigated.

The *AKT1* variant rs1130233 is a synonymous variant that has been linked to differential *AKT1* protein expression levels (Harris et al., 2005; Tan et al., 2008). In one study, patients who carried the risk allele had smaller cognitive changes than non-risk allele carrier patients when treated with lithium (Tan et al., 2011). The SNP rs10138227 is located in the 5'UTR promoter region of *AKT1* and could therefore likewise have an effect on the expression of *AKT1*.

The SNP rs34230287, located in the promoter, modulates *ARRB2* gene transcription and has been associated with response to methadone treatment (a drug used to treat opioid dependency) (Zhang et al., 2007; Oneda et al., 2010). The polymorphism rs1045280 is a synonymous SNP and has been associated with the development of Tardive dyskinesia (TD) in patients with schizophrenia (Liou et al., 2008).

3.7.3. Glutamate receptor, ionotropic, AMPA 2 (*GRIA2*)

Glutamate receptor, ionotropic, AMPA 2 (*GRIA2*) is one of a family of glutamate receptors that are sensitive to alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA), and function as ligand-activated cation channels. AMPA receptors mediate fast excitation in the CNS and play a key role in hippocampal synaptic long-term potentiation (LTP) and depression (LTD) (Din et al., 2010). These channels are assembled from four related subunits, *GRIA1-4*. *GRIA2* is essential for the channel to be permeable to calcium.

GRIA2 encodes for *GLUR2* subunits and is located on chromosome 4q32-33. This region has previously been associated with BPD and schizophrenia (Ekholm et al., 2003; McInnis et al., 2003). *GRIA2* is down-regulated by chronic lithium treatment in human neuronal cell lines (Seelan et al., 2008) and chronic lithium treatment decreases the levels of *GluR2* in hippocampal neurons (Du et al., 2008). SNPs in the region in which *GRIA2* is located, showed the strongest association with lithium response in a GWAS study, although genome-wide significance was not reached (Perlis et al., 2009). A SNP in *GRIA2*, rs3813296, has been associated with improvement in PANSS negative scores, a measure of response to antipsychotics (Crisafulli et al., 2012). In this study the synonymous SNP, rs4302506, will be investigated. This SNP has previously been investigated for association with BPD (Chiesa et al., 2012a), response to antipsychotics (Crisafulli et al., 2012), as well as response to treatment in major depressive disorder (MDD) (Chiesa et al., 2012b). An association was found between rs4303506 and age of onset of depression (Chiesa et al., 2012b).

3.7.4. Peroxisome proliferator-activated receptor gamma, co-activator 1 alpha (*PPARGC1α*)

Peroxisome proliferator-activated receptor (PPAR) gamma, co-activator 1 alpha (*PPARGC1α*) is located on chromosome 4p15 and encodes for a transcriptional co-activator, PGC-1 α , which regulates genes involved in energy metabolism. PGC-1 α , can interact with and regulate CREB, as well as increasing the transcriptional activity of thyroid hormone receptor and several nuclear receptors, including PPAR- α and PPAR- γ . Through the action of its targets it regulates mitochondrial function, oxidative stress, gluconeogenesis and lipogenesis (Puigserver et al., 1998; Wu et al., 1999; Herzig et al., 2001; Puigserver and Spiegelman, 2003).

Lithium treatment inhibits *PPARGC1 α* expression in human neuronal cell lines (Seelan et al., 2008) and to almost undetectable levels in brown preadipocytes during brown fat differentiation. This could explain disturbances in adipose tissue accumulation and weight gain experienced by patients on lithium treatment (Rodríguez de la Concepción et al., 2005).

The first polymorphism in *PPARGC1 α* to be investigated is the common nonsynonymous Gly482Ser polymorphism, rs8192678. This SNP has been associated with obesity (Ridderstråle et al., 2006), weight gain (Deeb and Brunzell, 2009), type 2 diabetes mellitus (Kunej et al., 2004; Barroso et al., 2006; Yang et al., 2011), non-alcoholic fatty liver disease (Yoneda et al., 2008) and insulin sensitivity (Stefan et al., 2007) amongst other phenotypes. The second SNP to be investigated, rs2279525, is located in the 5' UTR of *PPARGC1 α* and may influence protein expression, although the exact function is unknown.

3.8. Aim of this study

The aim of this pilot study was to identify suitable candidate genes and investigate variants in these genes for association with interindividual susceptibility to developing side effects on lithium treatment in a South African cohort of BPD type I patients.

Objectives:

- To collect data regarding twenty of the most common side effects reported in lithium-treated BPD type I patients
- To genotype the cohort for variants in the candidate genes, *GSK3B*, *AKT1*, *ARRB2*, *GRIA2* and *PPARGC1α*.
- To determine the association of these genetic variants with the type and frequency of side effects experienced.

Chapter 4. Materials and Methods

4.1. Cohort selection

The “Genetics of BPD” project was first approved at the University of Cape Town (UCT) in 1996 (HREC REF 182/96). Currently, the cohort consists of 883 individuals from 185 families. Other projects linked to this initiative, which have already received ethical approval, include *Presentation and Risk Factors in the Psychobiology of Psychosis* and *Neuroimaging: The Effects of Mindfulness Training in Bipolar Disorder*. Ethics approval for this study was provided by the Human Research Ethics Committee of UCT (Appendix A: HREC REF: 431/2012).

The patients in the cohort are diagnosed using the Structured Clinical Interview for DSM Disorders (SCID), which includes information on the response phenotype to all current and past treatments. All samples and patient information are collected according to the Declaration of Helsinki (World Medical Association, 2008). The patients are routinely followed up to update SCID data.

For the present study, patients on the Division of Human Genetics database with a diagnosis of BPD type I, that are known to have been treated with lithium, were retrospectively contacted. A side-effect questionnaire (Appendix C) similar to that used by Ghose (1977) was applied to determine their side-effect profile. A total of twenty side effects were assessed and rating was carried out according to a three-point rating scale: 0 = none, 1 = mild, 2 = moderate, 3 = severe. The questionnaire further addressed questions regarding lithium dose, time on lithium treatment, adherence to medication, as well as the number and type of other psychotropics prescribed to the patient, while on lithium treatment. Lastly patients were also asked whether they were ever advised to discontinue lithium treatment by their doctor and why. All questionnaires were conducted telephonically by a registered psychiatric nurse within the Division of Human Genetics at UCT.

Further patient recruitment was conducted at Valkenberg Psychiatric Hospital, Cape Town. Patients with a diagnosis of BPD type I (as diagnosed by a registered psychiatrist) and a history of treatment with lithium were approached for inclusion in the study. The side-effect questionnaire was conducted by either a project researcher or a psychiatric nurse. Interviews were conducted in either English or Afrikaans, except for one Xhosa patient for whom a translator was used.

Informed consent for DNA analysis and storage (Appendix B), as well as access to confidential medical records, was obtained from all patients, after which DNA was isolated from whole blood for further analysis. When a blood sample could not be obtained, a saliva sample was taken using the Oragene-DNA (OG-500) kit (DNA Genotek Inc., Canada) and DNA was isolated for further analysis. DNA samples are coded anonymously and stored in the molecular genetics laboratory of the Division of Human Genetics at UCT.

4.2. DNA isolation

Genomic DNA was isolated from peripheral blood lymphocytes according to the salting-out method by Gustafson and colleagues, a detailed protocol of which is provided in Appendix D (Gustafson et al., 1987). Following cell lysis and protein precipitation, ethanol precipitation of the DNA was performed. Thereafter, the DNA was rehydrated and stored.

The DNA isolation protocols were completed by a research assistant from the Division of Human Genetics. Each DNA sample was assigned a laboratory reference code to ensure confidentiality, and patient and family information were recorded on the database. One DNA sample is routinely archived at -80°C for long term storage and another at -20°C for short term storage. The latter was used for this study, unless that was depleted, in which case the other sample was retrieved from long term storage.

4.3. Concentration and integrity of isolated DNA

The quality of each of the DNA samples was determined by analysing the concentration and purity of the stock samples using the NanoDrop 1000 spectrophotometer (NanoDrop Technologies, USA).

Aliquots of the DNA stock samples were diluted with distilled water (dH₂O) to working concentrations of 100 ng/μl that were stored at 4°C for the duration of the research project. The DNA integrity was determined by electrophoresis of 100ng of diluted DNA combined with 5 μl loading buffer (Fermentas Life Sciences, Burlington, Canada). The DNA was electrophoresed at 160 V for approximately 20 minutes (min) through a 1% agarose gel (Appendix G). Each agarose gel contained 30ng of ethidium bromide (EtBr) (Sigma, England), a DNA intercalating agent, to enable visualisation. The DNA was visualised under UV light on the UVIPro UVIGold transilluminator (UVitec Limited, UK).

High molecular weight DNA appears as a bright solid band closest to the loading well, at the top of the agarose gel. DNA fragments of lower molecular weight, in which double-stranded breaks have occurred, migrate further than fragments of higher molecular weight. DNA which appears as a smear on the gel indicates a degraded sample.

4.4. Candidate gene and SNP selection

Candidate genes were selected based on thorough literature searches and biological databases queries. Genes were considered candidates if they were reported to be up- or down-regulated by lithium treatment, and if they had evidence of biological function in tissues involved in lithium-induced side-effects. SNPs in candidate genes were selected based on known functional properties, as published in the literature. SNPs were excluded if they had a minor allele frequency below 0.05. The genetic variants investigated in this study as well as the genotyping method used are indicated in Table 4.1.

Table 4.1: List of genes, SNPs and respective genotyping method

Gene	Chromosomal Location	NCBI RefSNP Identifier	Genotyping Method
GSK3B	3q13	rs334558	SNaPshot PCR
		rs6438552	SNaPshot PCR
		rs3755557	RFLP
AKT1	14q32	rs1130233	SNaPshot PCR
		rs10138227	TaqMan®
ARRB2	17p13	rs34230287	SNaPshot PCR
		rs1045280	SNaPshot PCR
GRIA2	4q32	rs4302506	TaqMan®
PPARGC1A	4p15	rs8192678	TaqMan®
		rs2279525	TaqMan®

4.5. Polymerase Chain Reaction (PCR)

4.5.1. External Primer design

The sequences containing the SNP of interest in the candidate genes were amplified using Polymerase Chain Reaction (PCR). External primers, complementary to regions flanking the sequence of interest, were designed for PCR amplification. The sequences of each of the candidate genes (*GSK3B*, *AKT1*, *ARRB2*, *GRIA2* and *PPARGC1A*) were obtained from NCBI (<http://www.ncbi.nlm.nih.gov>) and Ensembl (<http://www.ensembl.org/index.html>) databases. Gene sequences were then annotated using the ANNOTV9 annotation program (Rebello, 2006, Division of Human Genetics, UCT, Personal communication), which utilises Perl scripting language (Wall, 1994). The genes are annotated to specify the location of exons, start/stop codons, coding sequence and SNPs.

Each sequence, including approximately 400 base pairs (bp), on either side of the SNP locus was submitted in FASTA format to the Primer3 primer selection program (<http://frodo.wi.mit.edu/primer3/>) (Rozen and Skaletsky, 1999). The conditions for primer selection were: primer length between 18 and 23 bp, the melting temperature of the primers between 50° and 65°C, GC content between 45% and 55%, a maximum difference in melting temperature of 3°C between the primer pair and an amplicon size larger than 100 bp.

The primer pairs obtained from Primer3 were further assessed using the OligoCalculator program (<http://www.pitt.edu/~rsup/OligoCalc.html>) and the Integrated DNA Technologies (IDT) OligoAnalyzer version 3.1 web-based tool (<http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx>). Using OligoCalculator the length, melting temperature (T_m), percentage GC content and molecular weight (in Daltons, g/M) were determined. IDT OligoAnalyzer was used to analyse each primer pair for self-complementarity (hairpin formation), homodimer formation (the ability of the primer to self-dimerise) and heterodimer formation (dimer formation between the forward and reverse primers).

The primers were then submitted to the NCBI PrimerBLAST program (<http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>). The Basic Local Alignment Search Tool (BLAST) compares nucleotide or protein sequences to sequence databases and determines the statistical significance of similarity between aligned sequences. PrimerBLAST is therefore used to determine whether the primer pair is complementary to regions of the human genome other than the region of interest.

The primers were synthesised by the Department of Molecular and Cell Biology at UCT, using the Oligo 1000M DNA Synthesiser v4.20. All stock primer solutions were diluted in dH₂O to working concentrations of 20 micromolar (μM) and stored at 4°C. External primers for the PCR amplification reaction of rs334558, was previously designed (Dalvie, unpublished data, 2011) and synthesised by the Department of Molecular and Cell Biology at UCT. The primer sequences used to amplify the candidate SNPs are shown in Table 4.2. The table also includes the primer length, the size of the DNA fragment expected to result from PCR amplification and the optimal annealing temperature (Ta) determined through a temperature gradient experiment.

4.5.2. PCR optimisation

Prior to amplification of the selected SNPs, each PCR was optimised for annealing temperature, cycling conditions and concentration of reagents. A temperature gradient experiment, in which the Ta is varied over 12 reactions, was performed to determine the optimal annealing temperature (Ta). An example of the result of a temperature gradient experiment is shown in Appendix E.

The PCR reaction (Appendix H) included 100 ng DNA template, 10 picomoles (pmol) of each primer (forward and reverse), 200 μM of each deoxyribonucleotide triphosphate (dNTP) (Bioline, UK), 0.1 unit (U) GoTaq DNA Polymerase (Promega, USA), 1x Colorless GoTaq Reaction Buffer (Promega, USA) and dH₂O to make up the final reaction volume of 25 μl. Based on the Ta for the primer pairs and the amplicon size, the amplification of rs34230287 and rs1130233 could be run in one multiplex PCR reaction. A negative (no template) control, in which the DNA template is substituted with distilled water, was included in every PCR experiment, which allows one to control for contamination of reagents.

The standard cycling conditions for a PCR was 94°C for 3 min, and then repeated 30 cycles with 94°C for 30 s, the optimal Ta for each primer set (Table 4.2) for 30 s and 72°C for 30 s, followed by an extension step at 72°C for 10 min. PCR reactions were performed on the MultiGene™ Gradient Thermal Cycler (Labnet International, Inc., USA). Once completed, PCR reactions were stored at 4°C until required for downstream processes. To determine whether amplification had occurred, PCR products were electrophoresed at 160 V for approximately 40 min through a 1% agarose gel. The first lane of the gel was loaded with 100bp molecular weight marker (MWM) or 1kb MWM (Fermentas Life Sciences, Burlington, Canada) to estimate the size of DNA fragments (Appendix F). The PCR products were visualised under UV light on the UVIPro UVIGold transilluminator (UVitec Limited, UK).

Table 4.2: External primer sequences for amplification of candidate SNP regions using PCR

Gene	SNP	Primer Direction	Primer Length	Primer Sequence (5'→3')	Ta (°C)	Amplicon Size (bp)
<i>GSK3B</i>	rs334558	Forward	22	CGATTCCCAGACGCCTGTTACG	59	302
		Reverse	18	TCAGGAAGTGTCCGCGCT		
	rs6438552	Forward	21	CTTTTGGTGCCTTCTTAGGTG	59	286
		Reverse	20	CGAAACATTGGGTTCTCCTC		
	rs3755557	Forward	23	GTCCTTCATCAGTGTTCAAAGC	59	250
		Reverse	20	CCAGCGTCCATTGTTCTACC		
<i>AKT1</i>	rs1130233	Forward	21	ACTTGTTCTGCTGAGTTAGG	62	419
		Reverse	22	AGTCTGTGATCTTAATGTGCCC		
	rs10138227	Forward	22	TCTGAACCTCTCTTGTCTCCA	60	388
		Reverse	20	CAGCCCCTTGACTTCTTTG		
<i>ARRB2</i>	rs34230287	Forward	23	GTGAATAGCATAACATAGGCACG	62	494
		Reverse	21	CAACCCCTTGAAACCGATTGG		
	rs1045280	Forward	20	CAGTGACCAGGTATCTCCCA	59	571
		Reverse	22	TGAAATCCAGCCCTTGTGAAG		
<i>GRIA2</i>	rs4302506	Forward	21	GCACATCAACAAACCTAGCAG	60	313
		Reverse	18	TGCCCACCACTACACCTG		
<i>PPARGC1A</i>	rs8192678	Forward	20	CAGGGGCAGATTTGTTCTTC	59	410
		Reverse	20	GATTTGGGTGGTGACACAGA		
	rs2279525	Forward	20	CTTTTGGCCCTGGATTGTTG	59	549
		Reverse	20	CCCTCTGCTGCTTTGAAT		

4.6. SNaPshot™ PCR

SNaPshot™ PCR is a SNP genotyping method based on basic PCR amplification, the basic principle of which is demonstrated in Figure 4.1. The method requires the use of SNaPshot™ multiplex ready reaction mix (Applied Biosystems, CA, USA), which contains reaction buffer, fluorescently-labelled dideoxynucleotide triphosphates (ddNTPs), and AmpliTaq polymerase. Internal primers were designed to bind directly 5' or 3' of the SNPs of interest in *ARRB2* (rs34230287, rs1045280), *GSK3B* (rs3755557, rs6438552) and *AKT1* (rs1130233). An internal primer, for rs334558 in *GSK3B*, was previously designed (Dalvie, unpublished data, 2011).

The internal primer anneals to its complementary regions in PCR products of the regions of interest. It is then extended through the incorporation of a differentially-labelled ddNTP, complementary to the SNP of interest, the reaction of which is catalysed by *Taq* polymerase (ABI Prism SNaPshot™ Multiplex Kit Protocol, Applied Biosystems). Since ddNTPs lack a 3'-hydroxyl group, their incorporation into the sequence results in sequence termination. The SNaPshot reaction is resolved through capillary electrophoresis on an ABI genetic analyser and the alleles of every SNP are represented as differently coloured peaks. The ddNTPs are labelled as follows: ddATP – dR6G (green), ddCTP – dTAMRATM (black), ddGTP – dR110 (blue) and ddTTP – dROXTM (red).

4.6.1. SNaPshot PCR Internal Primer Design

Internal primers, that bind directly 3' or directly 5' to the SNP of interest, were designed using the same criteria as those for external PCR primers (section 4.5.1). One primer (either forward or reverse) was designed for every SNaPshot PCR reaction. In order to multiplex the SNaPshot reaction, the primers had to differ in length to prevent overlap in the SNaPshot products. Random, non-complementary tails were added to generate primers of different lengths. A local BLAST search, using the sequence alignment program, BioEdit Sequence Alignment Editor 7.0.0 (Hall, 1999), was performed to ensure that the primers would not hybridise to each other. The primer sequences and their resultant coloured peaks are indicated in Table 4.3.

Internal primers were synthesised by the Department of Molecular and Cell Biology at UCT, using the Oligo 1000M DNA Synthesiser v4.20. Primers were diluted in dH₂O to working concentrations of 20 µM and stored at 4°C. Working solutions of 20 µM was made from 100 µM stock solution through the addition of dH₂O.

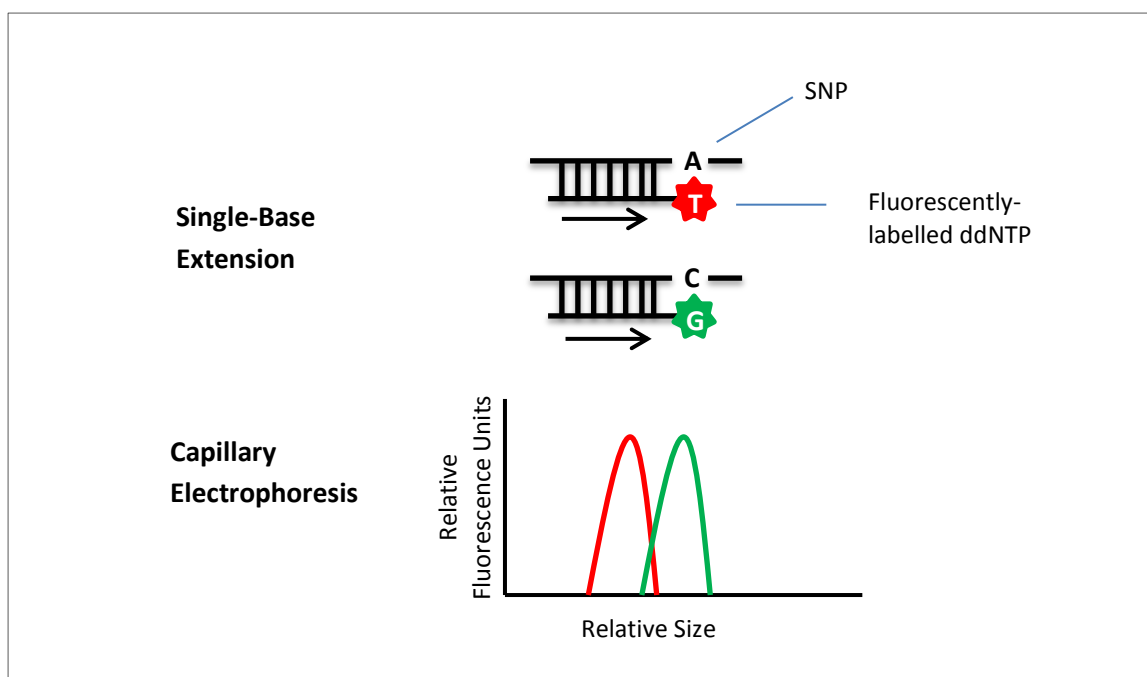


Figure 4.1: Illustration of the SNaPshot PCR principle. Internal primers are extended through the incorporation of fluorescently-labelled dideoxynucleotide triphosphates (ddNTPs) directly at the SNP of interest. Following capillary electrophoresis, the genotype of the particular SNP is visible as coloured peaks. In this figure the SNaPshot PCR genotyping of a TG heterozygote sample is demonstrated.

4.6.2. FastAP™ and *ExoI* Clean-up of PCR Products

Prior to SNaPshot PCR genotyping, excess dNTPs and unincorporated primers had to be removed to prevent interference with the SNaPshot genotyping reaction (ABI PRISM® SNaPshot™ Multiplex Kit Protocol, Applied Biosystems). FastAP™ (Fermentas Life Sciences, Burlington, Canada), is a thermo-sensitive alkaline phosphatase which catalyses the release of 5'- and 3'- phosphate groups from DNA. Exonuclease I (*ExoI*) degrades single-stranded DNA in a 3' to 5'-prime direction, thereby releasing deoxyribonucleoside 5'-monophosphates in a stepwise manner, while leaving 5'-terminal dinucleotides intact. This promotes the degradation of excess single-stranded primers (Lee et al., 2002).

The FastAP™ and *ExoI* purification process was carried out on a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, CA, USA). Thermal cycling was performed at 37°C for 60 minutes, followed by a 15 minutes at 75°C. The FastAP™ and *ExoI* reaction contained: 5 µl PCR product, 1 U FastAP™ (Fermentas Life Sciences, Burlington, Canada), 2 U *ExoI* (Fermentas Life Sciences, Burlington, Canada) and the volume was made up to a total of 20 µl using dH₂O.

Table 4.3: Internal primer sequences for genotyping of SNPs using SNaPshot PCR

Gene	SNP	Primer Direction	Expected alleles	Sequence	Primer length	Peak Colours
<i>AKT1</i>	rs1130233	Reverse	C/T	GTCCTCGGAGAACACACG	18	Black/Red
<i>ARRB2</i>	rs34230287	Reverse	A/G	*aaaaCGCCCGCCTTCCAGG	19	Green/Blue
<i>ARRB2</i>	rs1045280	Forward	C/T	aGTACACCATAACCCCACTGCTCAG	25	Black/Red
<i>GSK3B</i>	rs6438552	Reverse	A/G	ggcggcAATGTAACTGTA ACTATCTCT	28	Green/Blue
<i>GSK3B</i>	rs334558	Forward	T/C	ttagttagttagttagtaTCCTCACACAGAGCAGC	35	Black/Red

*Lowercase sequences indicate non-complementary tails

4.6.3. SNaPshot PCR Conditions

The SNaPshot PCR reaction was performed on GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, CA, USA). Cycling conditions consisted of three steps (96°C for 10 s, 50°C for 5 s and 60°C for 30 s) repeated for 25 cycles. A master mix was made up with SNaPshot™ Multiplex Ready Reaction Mix (Applied Biosystems, CA, USA), internal primers and dH₂O (Appendix H). The master mix was aliquoted and 5 µl cleaned-up PCR product was added to each well. Multiplex SNaPshot PCR reactions were performed by including primers for two or more SNPs. For each SNaPshot reaction, a negative water control was included, to ensure that the results obtained were not due to contamination.

Following the SNaPshot PCR reaction, one unit of FastAP™ was added to the SNaPshot PCR products and the samples were loaded into the GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, CA, USA). Thermal cycling was performed at 37°C for 60 min, immediately followed by 15 min at 75°C. This was to ensure that all unincorporated ddNTPs were removed from the reaction, to prevent the co-migration of the ddNTPs with the products of interest and interference with the genotyping results (ABI PRISM® SNaPshot™ Multiplex Kit Protocol, Applied Biosystems).

4.6.4. Capillary Electrophoresis on ABI PRISM

The purified SNaPshot products were subjected to capillary electrophoresis on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, CA, USA). The SNaPshot product (0.8 µL), together with 9 µL Hi-Di™ formamide (Applied Biosystems, CA, USA) and 0.2 µL GeneScan™120 Liz™ Size Standard (Applied Biosystems, CA, USA) were loaded into a 96-well microtitre plate (Axygen Scientific, USA). Hi-Di is a highly deionised formamide that denatures the double-stranded DNA and acts as a buffer to stabilise the single strands of DNA. The size standard allows sizing based on the mobility of unknown DNA fragments relative to fragments of known size in the size standard. Capillary electrophoresis was performed on the ABI Prism 3100 Genetic Analyzer (Applied Biosystems, CA, USA).

Raw data was collected by the ABI Prism 3100 Genetic Analyzer Data Collection software (version 1.1) (Applied Biosystems, CA, USA) and GeneMapper® Software (Applied Biosystems, CA, USA) was used to analyse the raw data and size the DNA fragments.

4.7. Restriction Fragment Length Polymorphism (RFLP) Analysis

Restriction endonucleases (REs) are bacterial enzymes that cleave the sugar-phosphate backbone of DNA at specific recognition sites. REs are used to perform Restriction Fragment Length Polymorphism (RFLP) analysis, in order to genotype a sample for the presence of a polymorphism or mutation. RFLP analysis can be used for genotyping if the presence of the particular polymorphism or mutation creates or destroys a RE recognition site. The number of fragments present after RE digestion will, therefore, differ depending on the alleles present in the sample.

The SNP rs3755557 in *GSK3B* was genotyped using RFLP analysis. Using Webcutter 2.0 (Heiman, 1997) the RE, *Tru1I*, with the restriction site 5' T/TTAA 3', was identified as an appropriate RE for RFLP analysis of this variant. The presence of the rs3755557 T allele abolishes a restriction site and digestion of the 250 bp PCR fragment results in two bands (165 and 85 bp) compared to the three bands (165, 57 and 28 bp) obtained when the A allele is present.

Prior to RE digestion of the PCR products, the digest reaction was optimised in terms of incubation time and concentration of reagents. The RE digest reaction was made up to a total volume of 20 µl. A standard reaction contained 10 µl PCR product, 1x Buffer R (Fermentas Life Sciences, Burlington, Canada), 2 Units *Tru1I* (Fermentas Life Sciences, Burlington, Canada) and was made up to the final volume with dH₂O.

The RE reaction was incubated for two hours at the optimum temperature of 65°C on a heating block, allowing for complete RE digestion of the PCR products. The digest products were electrophoresed at 120 V for approximately 50 min through a 4% agarose gel and visualised under UV light. An undigested PCR product was electrophoresed alongside the digested products for comparison, as well as a HyperLadder™ 50bp (Bioline, UK) for fragment size estimation (Appendix F).

4.8. TaqMan® SNP Genotyping Assay

The TaqMan® SNP Genotyping Assay (Applied Biosystems, CA, USA), also known as the “5'-nuclease allelic discrimination assay”, was used to genotype SNPs in *AKT1* (rs10138227), *GRIA2* (rs4302506) and *PPARGC1a* (rs8192678, rs2279525). This method uses two allele-specific TaqMan® minor groove binder (MGB) probes and a PCR primer pair to detect the SNP of interest in one thermal cycling reaction. Each allele-specific MGB probe is labelled with either 2'-chloro-7'-phenyl-1,4-dichloro-6-carboxyfluorescein (VIC) or C-carboxyfluorescein (FAM) fluorescent reporter dye and has a fluorescence quencher attached to it.

During PCR amplification, the 5'-nuclease activity of *Taq* DNA polymerase cleaves the reporter dye (FAM or VIC) from a MGB probe that is completely hybridised to the DNA strand. Once it is separated from the quencher, the reporter dye fluoresces (Figure 4.2). When a single point mismatch is present between the probe and target DNA, as in the case of a SNP, the hybridisation of the probe to the DNA is less stable, thereby reducing the efficacy of the probe cleavage and quenching of the fluorescent reporter dye (Shen et al., 2009). When the levels of both fluorescent dyes are equal it indicates heterozygosity at a SNP, and where there is an increase in the levels of either FAM or VIC dye fluorescence, it indicates homozygosity for the FAM- or VIC-specific alleles.

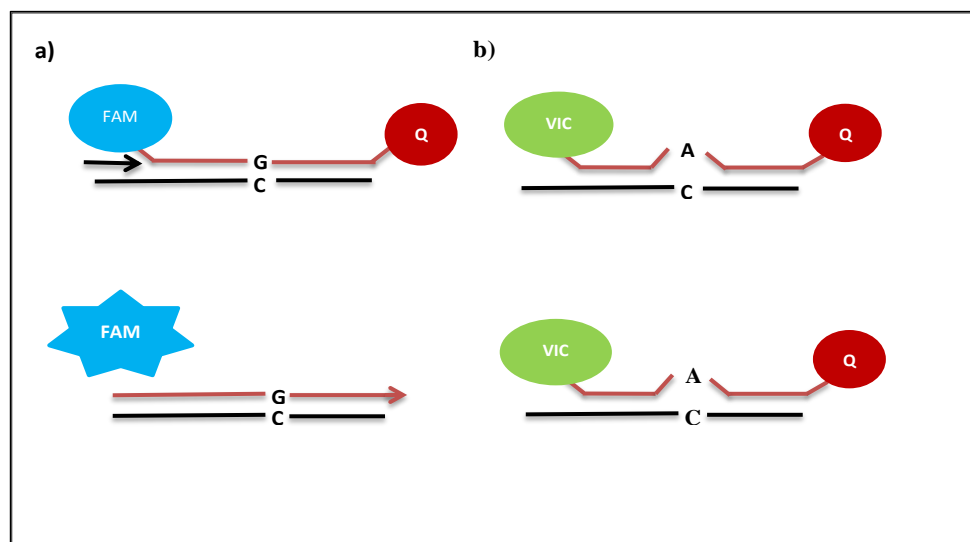


Figure 4.2: Taqman SNP genotyping reaction of a CC homozygote. a) When the MGB probe completely hybridises to the DNA strand, the reporter dye is cleaved from the MGB probe, the quencher (Q) is released and the reporter dye (FAM) fluoresces. b) When the MGB probe is not completely complementary to the DNA strand, the quencher remains attached to the reporter dye and it does not fluoresce.

4.8.1. TaqMan® SNP genotyping protocol

Pre-designed, validated TaqMan® SNP Genotyping Assays were ordered from Applied Biosystems, CA, USA. Each of the 40x SNP Genotyping Assays contain sequence-specific forward and reverse primers to amplify the region of interest, and two TaqMan® MGB probes, one labelled with VIC® dye to detect Allele 1 and the other with FAM™ dye to detect the Allele 2 sequence (Applied Biosystems, 2006). The assays are optimised and designed to work with a TaqMan® Universal PCR Master Mix (Applied Biosystems, CA, USA) at the same thermal cycling conditions.

The SNP genotyping assays were diluted to a 20x working stock with 1X TE buffer and aliquots were made for routine use to minimize freeze-thaw cycles. In a total reaction volume of 10 µl, 20 ng of genomic DNA were mixed with 5 µl TaqMan® Universal PCR Master Mix (Applied Biosystems, CA, USA), 0.50 µL of 20x TaqMan SNP Genotyping Assay and dH₂O. The TaqMan SNP genotyping reactions were aliquoted into wells of a Hard-Shell® PCR plate (Bio-Rad Laboratories, Inc., USA) and sealed with Microseal® “B” Adhesive Film (Bio-Rad Laboratories, Inc., USA). The cycle sequencing reaction was performed on a Bio-Rad CFX96 (Bio-Rad Laboratories, Inc., USA) using the recommended conditions: 95°C for 10 min, followed by 40 cycles of 92°C for 15 s and 60°C for 1 min.

Individual samples were assigned to a specific genotype by plotting the end-point relative fluorescence units (RFU) of the reporter dyes in each sample well on a Cartesian plot (also known as a scatter or cluster plot). A clustering algorithm in the Bio-Rad CFX96™ data analysis software (Bio-Rad Laboratories, Inc., USA) assigns individual sample data to a particular genotype cluster.

4.9. Genotype Validation

Cycle sequencing is a DNA sequencing technique based on traditional Sanger sequencing, in which chain terminating inhibitors are used (Sanger et al., 1977). Cycle sequencing is similar to a PCR, with the exception that fluorescently-labelled ddNTPs are incorporated in the reaction mix. The ddNTPs lack a 3'-hydroxyl group, therefore their incorporation into the sequence by DNA polymerase results in sequence termination. Each ddNTP is labelled with a differently coloured fluorescent marker to enable detection. Cycle sequencing was used to verify the genotyping results of SNaPshot PCR, RFLP and TaqMan SNP genotyping reactions.

A subset of samples was selected for sequencing based on genotyping results. One sample of all three possible genotypes (homozygous and heterozygous) was selected for each of the SNPs. PCR amplification was performed on the samples as previously described (section 4.5). PCR products were cleaned-up with FastAP™ and *ExoI* as described in section 4.6.2. Cycle sequencing was performed using the BigDye® Terminator (version 3.1) cycle sequencing kit (Applied Biosystems, CA, USA). The terminator mix contained the ddNTPs, as well as the thermo-stable DNA polymerase. The sequencing reaction included 1.5 µl PCR product, 0.5 µM of primer, 0.5 X terminator mix, and 1 X sequencing buffer. Sequencing reactions were made up to 20 µl with dH₂O.

All cycle sequencing reactions were performed on a GeneAmp® PCR System 9700 thermal cycler (Applied Biosystems, CA, USA). The cycling conditions consisted of an initial denaturation step at 95°C for 5 min, 30 cycles consisting of a denaturation step at 96°C for 10s, an annealing step at 50°C for 15 s and an extension step at 60°C for 4 min. Following cycle sequencing, an ethanol precipitation was performed (Appendix H) to remove any unincorporated ddNTPs which could interfere with base-calling. Following ethanol precipitation, the samples were re-suspended in 10 µl dH₂O, and capillary electrophoresis performed on an ABI Prism 3100 Genetic Analyzer (Applied Biosystems, CA, USA). The results were analysed using BioEdit Sequence Alignment Editor Version 7.0.0 (Ibis Biosciences, USA) (Hall, 1999).

4.10. Statistical Analysis

Statistical analysis was performed using Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) to calculate Hardy-Weinberg Equilibrium (HWE), while linkage disequilibrium analysis was performed using SHEsis (Shi and He, 2005). The statistical environment R version 2.15 (<http://www.R-project.org>) was used to create a Spearman correlation matrix of the relationship between side effects. All genotype-phenotype correlations and other analyses were performed using SPSS version 21 (SPSS IBM, New York, USA). Normality testing was performed using the Shapiro-Wilk normality test. A p-value <0.05 was considered statistically significant for all tests. No correction for multiple testing was performed as this was an exploratory pilot study. The reported side effects were grouped as absent (zero side effect reported) or present (mild, moderate or severe side effect reported). The total side effect frequency was calculated by summing the total number of “present” side effects in a patient.

The relationship between demographics (sex and ethnicity) and lithium dose, lithium level and total side effect frequency was assessed by the Mann-Whitney Test. The relationship between total side effect frequency and lithium dose and lithium level were assessed by Spearman correlation. Variables significantly associated with total side effect frequency were further investigated for association with individual side effects using Pearson’s Chi-squared test.

To assess the effect of other psychotropics on the side effect profile of patients, individuals on lithium monotherapy were compared to individuals using lithium in combination with other psychotropics. An Independent T-test was used to compare the total number of side effects experienced between these two groups. The contribution of the different types of other psychotropics (anticonvulsants, antidepressants and antipsychotics) on the presence or absence of individual side effects was determined through Chi-squared analysis.

The association of genetic polymorphisms in *AKT1*, *ARRB2*, *GSK3B*, *GRIA2* and *PPARGC1A* on the presence of each of the twenty side effects was investigated using a Chi-squared test. Significant results from the Chi-squared analysis were further assessed using logistic regression to determine whether the genetic associations remained significant after potential covariates (sex, age, ethnicity and the use of other psychotropics) were taken into account.

Backwards stepwise regression was applied using the likelihood-ratio test to eliminate variables from the model in an iterative process. The model is tested after the elimination of each variable to determine whether the model adequately fits the data. The analysis is complete when no more variables can be eliminated from the model. An Odds Ratio (OR) was calculated as a measure of risk. The corresponding 95% confidence interval (95% CI) was also calculated.

Chapter 5. Results

5.1. Cohort description and DNA extraction

The study cohort consisted of 105 individuals with a diagnosis of BPD I and a history of treatment with lithium. Of the cohort, 68 (64.8%) were female and 37 (35.2%) male; 38 (36.2%) were of Mixed Ancestry and 60 (57.1%) were Caucasian (represented in Figure 5.1). The average age (\pm SD) of the study participants at the time of interview was approximately 46 (\pm 11.68) years and ranged from 26 to 77 years. Seventy-two of the patients in the cohort were recruited previously and contacted telephonically to collect further information about side effects that were experienced while they were on lithium treatment. The remaining patients (n=33) were recruited from Valkenberg Psychiatric Hospital (Observatory, Cape Town).

A prerequisite of admission into the study was the availability of biological material from the research subjects. Generally, this was in the form of EDTA blood, which was processed to DNA using the salting-out method. DNA samples were quantified by means of spectrophotometry (NanoDrop Technologies, USA), and integrity determined by electrophoresis through a 1% agarose gel an example of which is given in Appendix D.

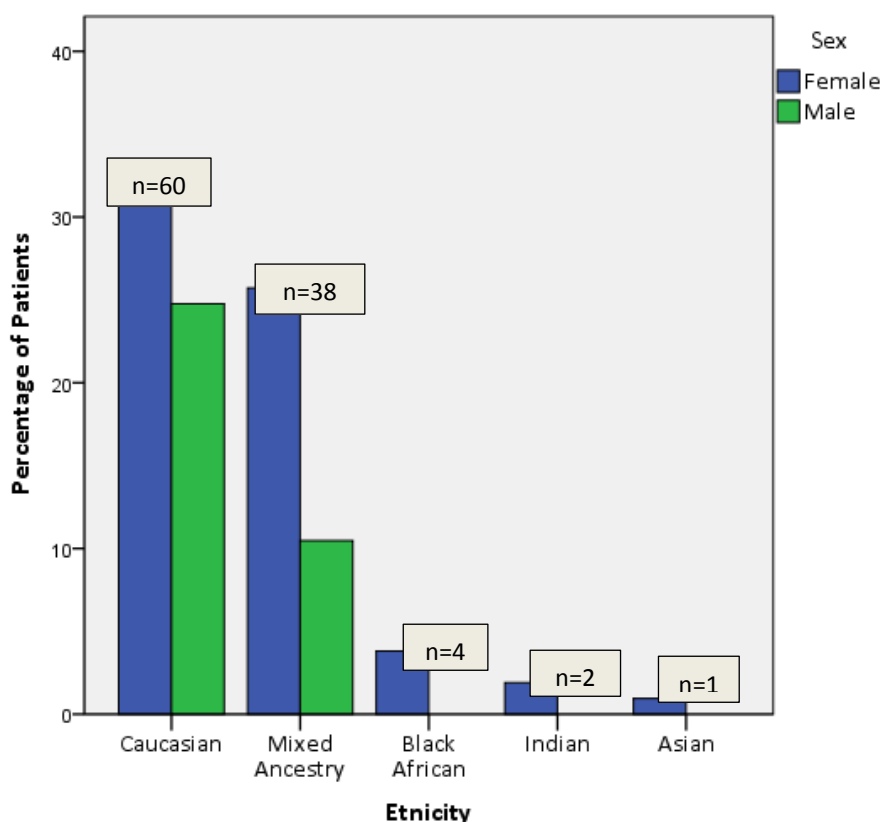


Figure 5.1 The distribution of the patient cohort (n=105) in terms of ethnicity and sex

5.2. SNP genotyping results

5.2.1. SNaPshot™ PCR reaction

Two multiplex SNaPshot PCR genotyping reactions were performed in order to save time and reagents. The first multiplex reaction (Group A) included rs34230287, rs1130233 and rs6438552 and the second (Group B) included rs334558 and rs1045280. The PCR products for the two SNaPshot reactions were multiplexed from the FastAP™ and *ExoI* clean-up stage.

The genotypes for five SNPs were therefore incorporated in two SNaPshot multiplex reactions. Chromatograms of Group A and B are given in Figures 5.2 and 5.3. In the chromatogram, homozygous genotypes are visible as a single, large peak. Heterozygous genotypes are indicated by two overlapping or adjacent peaks of a different colour.

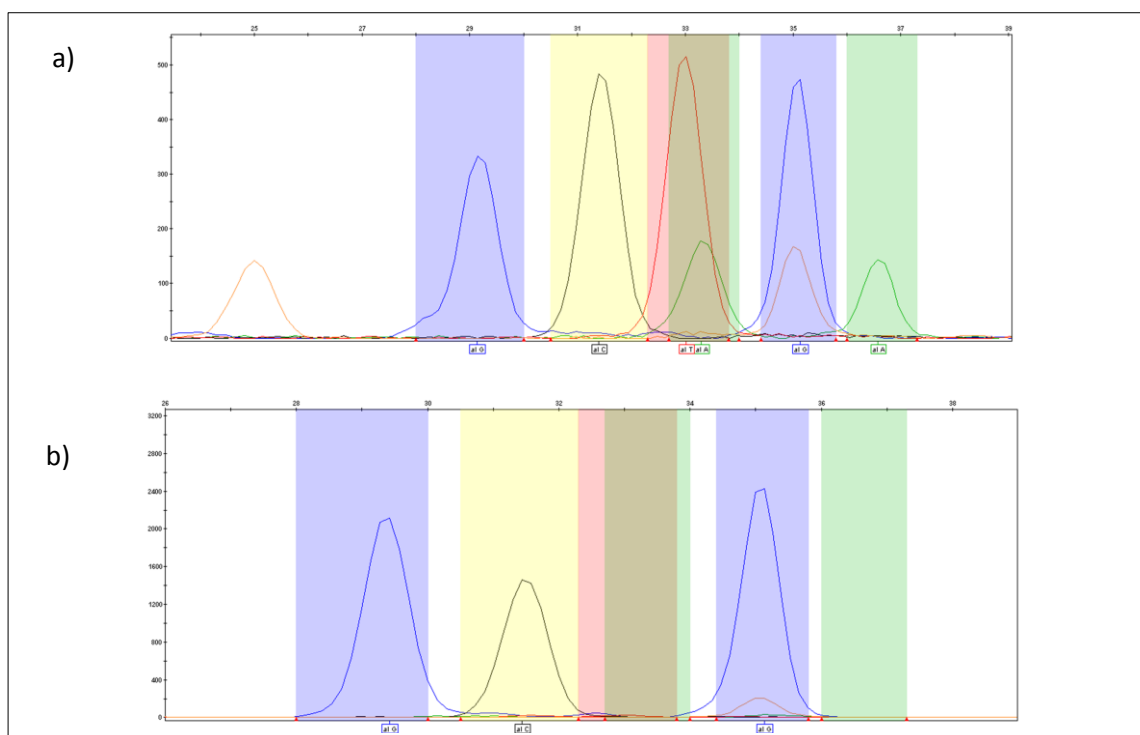


Figure 5.2: Chromatogram of SNaPshot results for Group A multiplex reaction. The X-axis represents the size or base position of the alleles and the Y-axis represents the relative fluorescent units. The coloured panes from left to right indicate the following alleles: blue pane – rs34230287 G allele, yellow pane – rs1130233 C allele, red pane – SNP 4 T allele, green pane – rs34230287 A allele, blue pane – rs6438552 G allele, Green pane – rs6438552 A allele. Orange peaks represent Genescan-120 Liz size standard. Genotypes in a): rs34230287 – heterozygote AG, rs1130233 – heterozygote CT, rs6438552 – heterozygote AG. Genotypes in b): rs34230287 – homozygous GG, rs1130233 – homozygous CC, rs6438552 – homozygous GG.

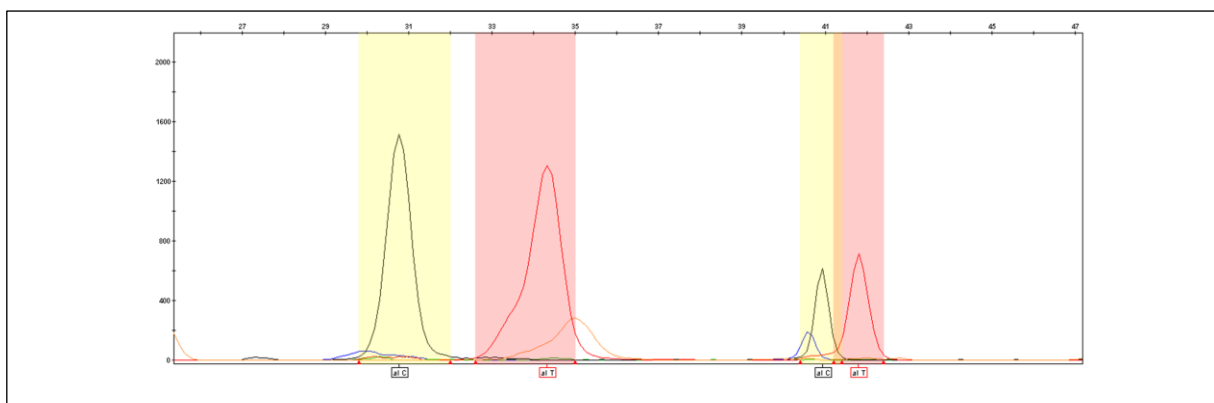


Figure 5.3: Chromatogram of SNaPshot results for Group B multiplex reaction. The X-axis represents the size or base position of the alleles and the Y-axis represents the relative fluorescent units. The coloured panes from left to right indicate the following alleles: yellow pane – rs1045280 C allele, red pane – rs1045280 T allele, yellow pane – rs334558 C allele, red pane – rs334558 T allele. Orange peaks represent Genescan-120 Liz size standard. Genotypes in this reaction: rs1045280 – heterozygote CT, rs334558 – heterozygote CT.

5.2.2. Restriction Fragment Length Polymorphism Analysis

Following PCR amplification, the SNP rs3755557 was genotyped by RE digestion. An example of the results obtained is shown in Figure 5.4. The 250bp PCR product was digested by *Tru1I* into 165bp, 57bp and 28bp fragments when an A allele was present, while the T allele resulted in digested fragments of 165bp and 85bp.

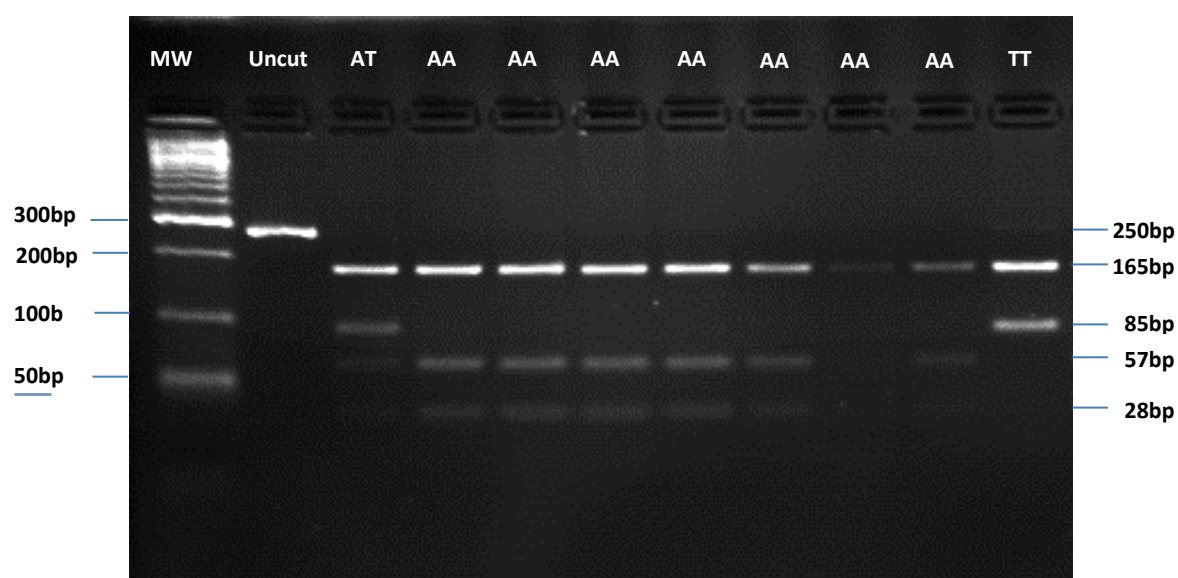


Figure 5.4: Representative RFLP SNP genotyping results for rs3755557 following *Tru1I* digestion showing AA, AT and TT genotypes. MWM indicates the 50 bp molecular weight marker: HyperLadder™ 50bp (Bioline, UK). An undigested PCR product was run with the products of every RE digest. All digest products were electrophoresed through 4% agarose gels stained with EtBr at 120 V for 60 min and visualised under UV light.

5.2.3. TaqMan® SNP Genotyping Reaction

Rs10138227 in *AKT1*, rs4302506 in *GRIA2*, and rs8192678 and rs2279525 in *PPARGC1A* were genotyped using pre-designed TaqMan® SNP genotyping assays. The results were analysed using Bio-Rad CFX96™ system software (Bio-Rad Laboratories, Inc., USA). The end-point RFU levels of the FAM and VIC fluorophores in every sample well were plotted on a Cartesian or allelic discrimination plot and RFU values were normalised relative to the negative controls, to assign a sample to a specific genotype (Figure 5.5).

FAM-labelled homozygous alleles are located in the top left quadrant, VIC-labelled homozygous alleles in the bottom right quadrant and heterozygotes are located in the upper right quadrant.

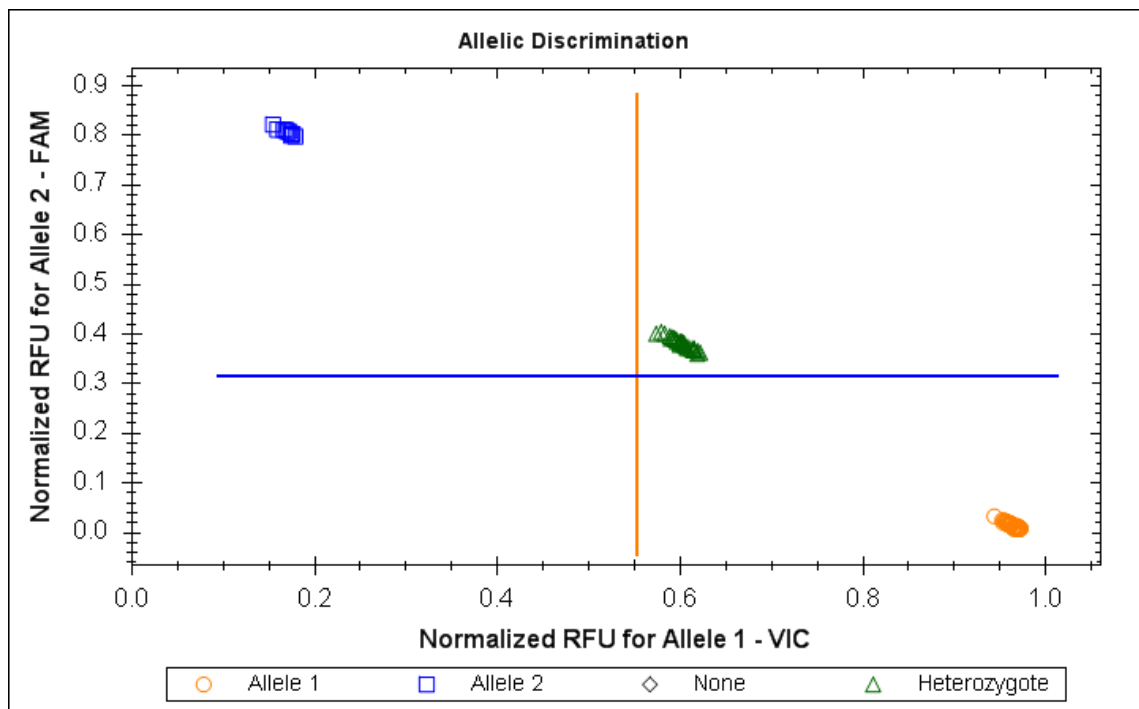


Figure 5.5: An allelic discrimination plot of rs8192678 in *PPARGC1A* following TaqMan® SNP Genotyping. The major allele (G allele) was detected by VIC-labelled probes and the minor allele (A allele) by FAM-labelled probes. The genotyping results are presented as allelic discrimination plots with the normalised relative fluorescent units (NRFU) of VIC on the X-axis and the NRFU of FAM on the Y-axis. Homozygotes for the VIC-labelled allele are located in the bottom right quadrant, homozygotes for the FAM-labelled allele in the upper left quadrant and heterozygotes in the top right quadrant.

5.2.4. Genotype Validation

The genotyping for all SNP results were validated by sequencing the PCR products of one sample from every genotype as determined by SNaPshot, TaqMan® or RFLP analysis. A total of 30 samples were therefore sequenced. Figure 5.6 represents a portion of the sequencing results of rs2279525 in *PPARGC1A*. The sample shown in figure 5.6 was homozygous for the G allele. The SNP is visible as blue peak (C allele) at position 95 (as indicated by the arrow), as the sequencing reaction was performed with the reverse primer. Figure 5.7 represents a heterozygous sample (C/T) for rs1045280 in *ARRB2*.

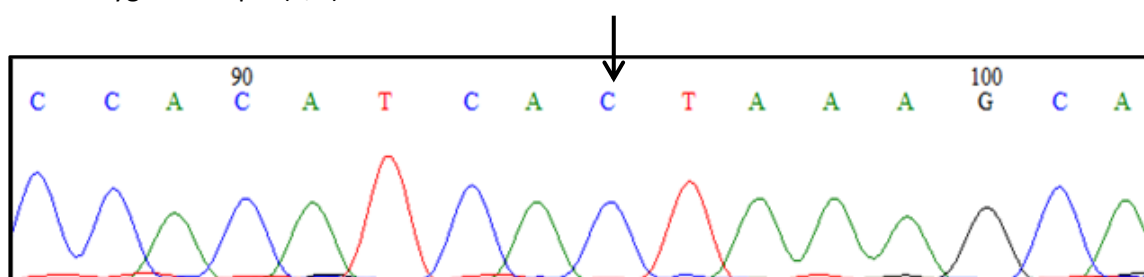


Figure 5.6: Sequencing results for rs2279525 in *PPARGC1A*. This sample is a homozygous for the G allele, seen here as a C allele, that is visible as a blue peak at position 95 (indicated with the arrow).

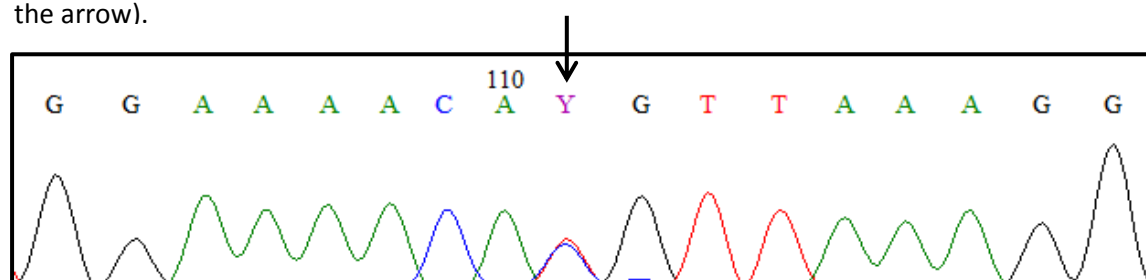


Figure 5.7: Sequencing results for rs1045280 in *ARRB2*. This sample is heterozygous (C/T) and the alleles are visible as a red and blue peak at position 111 (indicated with the arrow).

5.3. Genotype and allele frequencies

The genotype frequencies of the SNPs in *GSK3B*, *AKT1*, *ARRB2*, *GRIA2* and *PPARGC1A* are indicated in Table 5.1. HWE was calculated for the total cohort and the genotype frequencies of one SNP (rs334558) deviated from HWE ($p=0.049$). When analysing the HWE of the Caucasian and Mixed Ancestry populations alone, however, no SNPs deviated from HWE. A chi-squared test was conducted and genotype frequencies did not differ significantly between the Caucasian and Mixed Ancestry population groups at the $p=0.05$ level, except for the SNP rs334558 in *GSK3B*. The other population groups were not included in this calculation as there were only four Black African patients, two Indian and one Asian patient in the cohort.

Table 5.1: Overall and population-specific genotype frequencies of genetic polymorphisms in *GSK3B*, *AKT1*, *ARRB2*, *GRIA2* and *PPARGC1A*

Genotype	All patients Number (percentage)	Hardy Weinberg Equilibrium (p-value)	Population group		Population comparison p-value	Hardy Weinberg Equilibrium (p-value)	
			Caucasian	Mixed Ancestry		Caucasian	Mixed Ancestry
rs334558 in <i>GSK3B</i>	102		59	36			
CC	32 (31.37)	0.049	13 (22.0)	16 (44.4)	0.047	0.20	0.17
CT	41 (40.20)		24 (40.7)	13 (36.1)			
TT	29 (28.43)*		22 (37.3)	7 (19.4)			
rs6438552 in <i>GSK3B</i>	103		59	37			
CC	30 (29.13)	0.970	14 (23.7)	13 (35.1)	0.475	0.70	0.73
CT	51 (49.51)		31 (52.5)	17 (45.9)			
TT	22 (21.36)		14 (23.7)	7 (18.9)			
rs3755557 in <i>GSK3B</i>	102		59	36			
AA	71 (69.61)	0.258	43 (72.9)	24 (66.7)	0.542	0.81	0.23
AT	30 (29.41)		15 (25.4)	12 (33.3)			
TT	1 (0.98)		1 (1.7)	0 (0.0)			
rs1130233 in <i>AKT1</i>	102		58	37			
GG	55 (53.92)	0.649	26 (44.8)	25 (67.6)	0.081	0.59	0.87
GA	41 (40.20)		27 (46.6)	11 (29.7)			
AA	6 (5.88)		5 (8.6)	1 (2.7)			
rs10138227 in <i>AKT1</i>	105		60	38			
GG	78 (74.29)	0.998	41 (68.3)	31 (81.6)	0.275	0.54	0.32
GA	25 (23.81)		18 (30.0)	6 (15.8)			
AA	2 (1.90)		1 (1.7)	1 (2.6)			
rs34230287 in <i>ARRB2</i>	101		59	35			
CC	76 (75.25)	0.377	40 (67.8)	30 (85.7)	0.105	0.52	0.11
CT	22 (21.78)		18 (30.0)	4 (11.4)			
TT	3 (2.97)		1 (1.7)	1 (2.9)			

rs1045280 in <i>ARRB2</i>	102		59	36			
TT	56 (54.90)	0.664	35 (59.3)	19 (52.8)	0.164	0.51	0.20
TC	38 (37.25)		22 (37.3)	12 (33.3)			
CC	8 (7.84)		2 (3.4)	5 (13.9)			
rs4302506 in <i>GRIA2</i>	105		60	38			
CC	45 (42.86)	0.893	28 (46.7)	15 (39.5)	0.106	0.39	0.26
CT	47 (44.76)		28 (46.7)	15 (39.5)			
TT	13 (12.38)		4 (6.7)	8 (21.1)			
rs8192678 in <i>PPARGC1A</i>	104		58	38			
GG	61 (58.65)	0.595	29 (50.0)	26 (68.4)	0.203	0.99	0.44
GA	36 (34.62)		24 (41.4)	10 (26.3)			
AA	7 (6.73)		5 (8.6)	2 (5.3)			
rs2279525 in <i>PPARGC1A</i>	104		59	38			
AA	52 (50.0)	0.059	28 (47.5)	19 (50.0)	0.385	0.90	0.07
AG	37 (35.58)		25 (42.4)	12 (31.6)			
GG	15 (14.42)		6 (10.2)	7 (18.4)			

*Values in brackets indicate percentages

5.4. Linkage Disequilibrium Testing

Linkage disequilibrium (LD) analysis was performed for the three SNPs in the *GSK3B* gene, the two SNPs in *AKT1*, the two SNPs in *ARRB2* and the two SNPs in *PPARGC1A*. LD was calculated for the Caucasian and Mixed Ancestry population groups. Pairwise linkage disequilibrium coefficient (D') and correlation coefficient (r^2) values are reported in Table 5.2. The r^2 should be similar to D' values, as both are measures of linkage disequilibrium; however r^2 value are dependent on allele frequencies and these values were much lower than D' values. This discrepancy may be due to the small sample size and differences in minor allele frequency between SNPs. The r^2 is preferred by the HapMap project and were used in this study. According to this measure, no tight LD was observed between any of the SNPs and no haplotypes could be inferred.

If the D' value is taken in to account, however, the D' value of 1.000 indicated that the three SNPs in *GSK3B* (rs334558, rs375557 and rs643852) are in complete LD in Caucasians. In the Mixed Ancestry population, the D' (0.831) between rs334558 and rs643852 indicate tight, but not complete LD. A D' value of 0.998 indicated that rs10138227 and rs1130233 in *AKT1* were in tight LD in the Mixed Ancestry population, but LD was weaker in Caucasians (0.724).

Table 5.2: Linkage disequilibrium analysis of *GSK3B*, *AKT1*, *ARRB2* and *PPARGC1a*

Gene	SNP Pair	Caucasian		Mixed Ancestry	
		D'	R^2	D'	R^2
<i>GSK3B</i>	rs375557-rs334558	1.000	0.231	1.000	0.129
	rs375557-rs643852	1.000	0.160	1.000	0.129
	rs334558-rs643852	1.000	0.691	0.831	0.690
<i>AKT1</i>	rs10138227-rs1130233	0.724	0.045	0.998	0.026
<i>ARRB2</i>	rs34230287-rs1045280	0.710	0.346	0.012	0.000
<i>PPARGC1a</i>	rs2279525-rs8192678	0.832	0.128	0.415	0.022

5.5. Side effect frequencies

Data was collected regarding twenty common side effects that patients may experience while on lithium treatment. Patients were asked to rate the severity of a specific side effect on three-point rating scale: 0 = none, 1 = mild, 2 = moderate, 3 = severe

The median number of side effects reported was six (variance 13.013). A histogram of the distribution of side effect frequency is indicated in Figure 5.8. Three patients reported experiencing zero side effects while on lithium. The maximum number of side effects reported was nineteen. This patient was an outlier that was removed in further analyses as the individual reported experiencing nineteen of the twenty potential side effects, although (other than weight gain and thyroid problems), these were all reported to be mild.

The frequency and severity of the different side effects is shown in Figure 5.9. The most common reported side effect was polydipsia (75.2%), followed by a dry mouth (71.6%) and polyuria (60.4%). Over 40% of patients reported polydipsia to be severe. 54.4% of patients reported to have gained weight while on lithium treatment and 17.3% reported this as severe weight gain. Similarly, 54.4% of patients reported experiencing tremors. Tremor was mild in the majority (27.9%) of patients. The least common side effect was a congested (or stuffy) nose (7.7%).

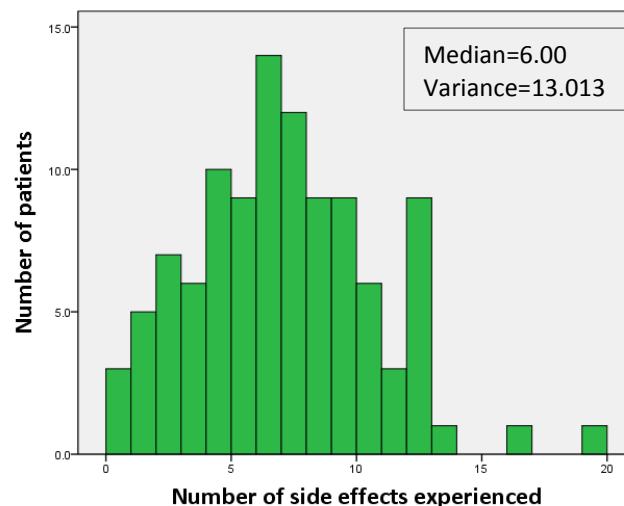


Figure 5.8: Distribution of the number of side effects reported by patients. The median number of side effects reported was 6 (Variance=13.013).

A skin problem was reported by 24% of patients. Twelve patients had acne, seven had extremely dry skin and three suffered from psoriasis. One patient suffered from *Hidradenitis Suppurativa* (skin disease that most commonly affects areas bearing apocrine sweat glands or sebaceous glands), two complained of consistent rash and one was diagnosed with dermatitis.

Of the cohort, three patients had to discontinue lithium treatment due to kidney problems, although the severity of kidney damage was not assessed. Lithium toxicity occurred in five patients, although it is unclear whether this was due to intentional or accidental lithium overdose. Severe weight gain was a further reason given for discontinuing lithium treatment, as well as feelings of being “numb” or “like a zombie”.

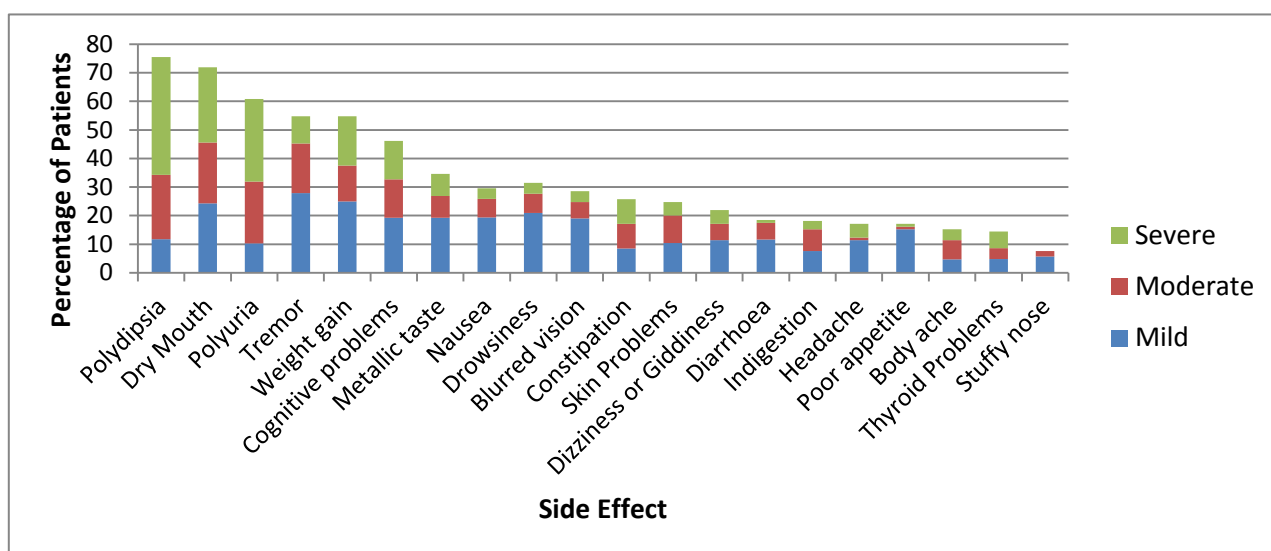


Figure 5.9: Incidence and severity of reported lithium-induced side effects.

5.5.1. Correlations between side effects

A Spearman's correlation was performed to assess the relationship between different side effects and to determine whether any side effects cluster together. There were no complete correlations between side effects, with the strongest correlation being between polyuria and polydipsia at 0.563 and between polydipsia and dry mouth at 0.535. Weak correlations were also observed between the gastrointestinal side effects. There was a correlation of 0.349 between indigestion and constipation and a correlation of 0.323 between indigestion and nausea. A correlation matrix is shown in figure 5.10 and a table with all correlation values are given in Appendix I.

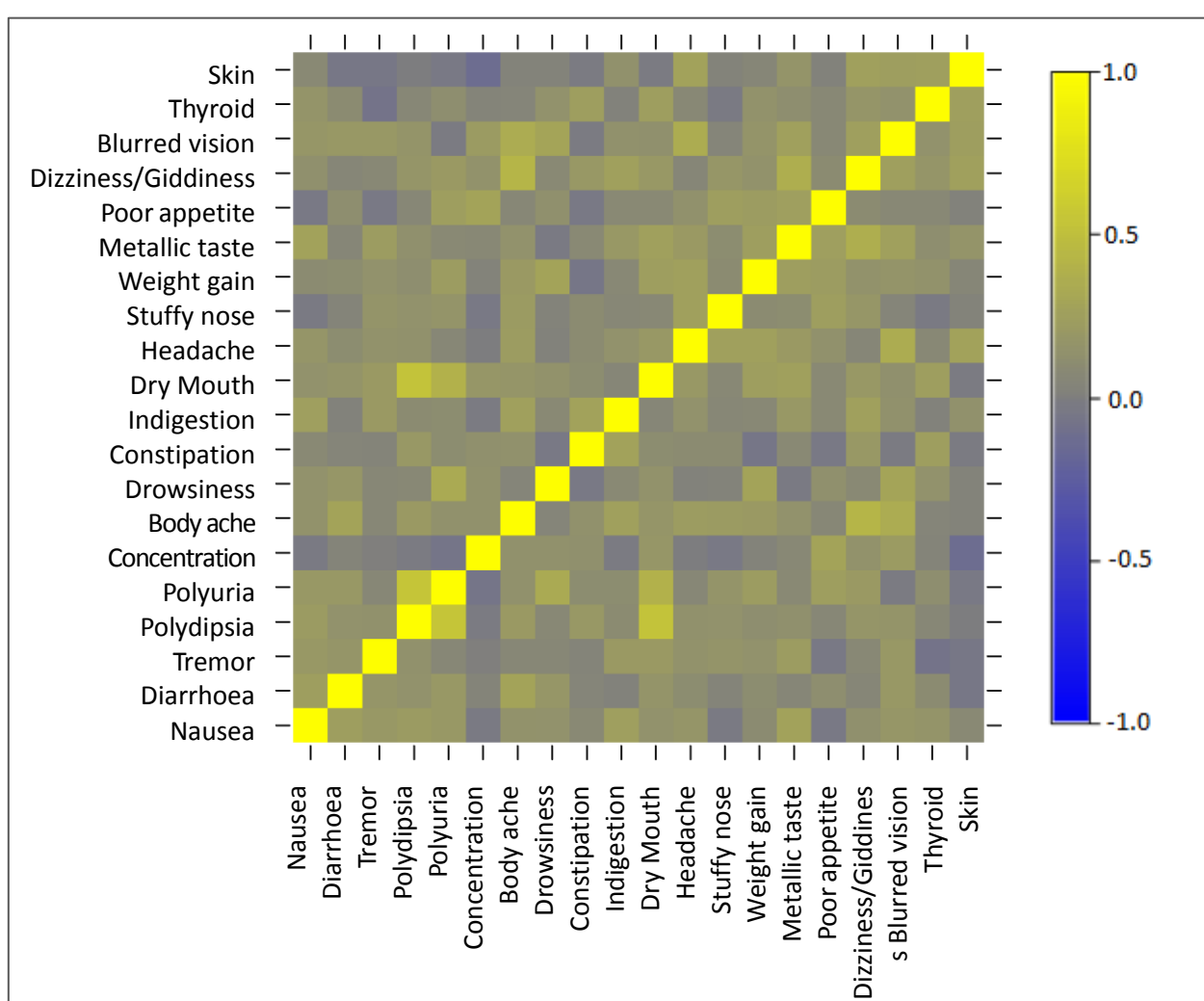


Figure 5.10: A Spearman's correlation matrix of investigated side effects. The strongest correlation is between polydipsia and polyuria at $r=0.56$.

5.5.2. Demographic and clinical variables

There was no significant correlation between lithium dose and the total side effect frequency in patients ($r^2=0.038$, $p=0.728$). The median prescribed daily lithium dose was 800 mg, with a significant difference between males and females ($p=0.030$). The median dose for men was 1000 mg and for females this was 750 mg lithium per day (Figure 5.11). There was no significant difference in the lithium dose between patients of Caucasian or Mixed Ancestry origin ($p=0.706$).

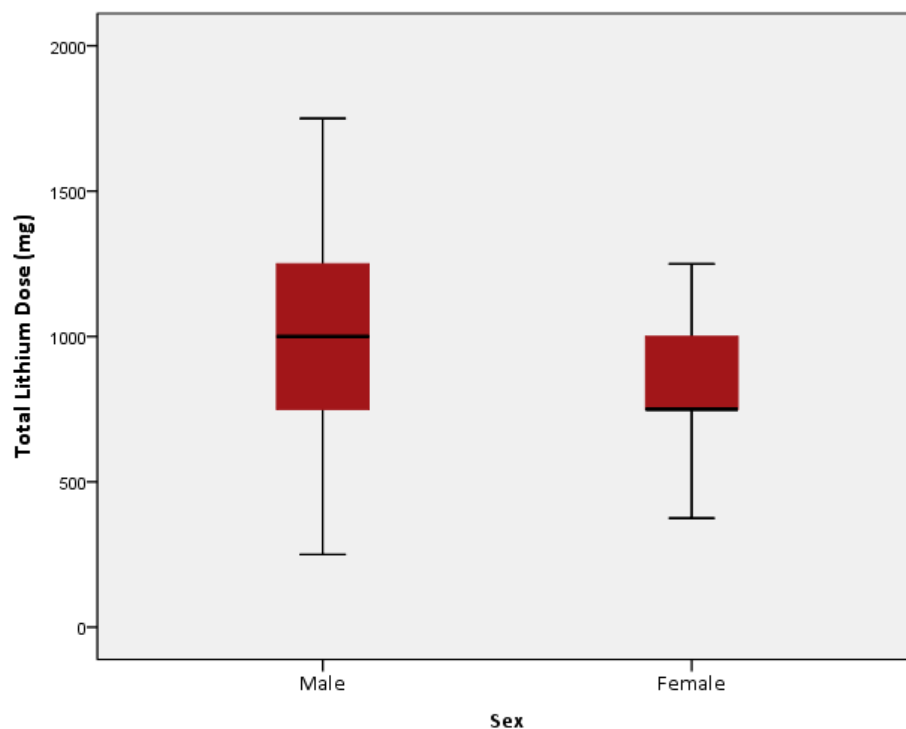


Figure 5.11: Distribution of lithium dose in males and females. The median dose for males is 1000mg per day and for females it is 750mg per day.

Information on lithium levels in blood was only available for 17 patients. There was no correlation between lithium levels and the number of side effects experienced ($r^2=0.346$, $p=0.147$). Patients were requested to report their treatment adherence, but all patients reported a 75-100% adherence rate and this information was therefore not taken into account in further analysis.

It was further investigated whether total side effect frequency differs in terms of demographics (sex and ethnicity) using a Mann-Whitney Test. A summary of the descriptive data is given in table 5.3. There was a significant difference in the total number of side effects experienced between males and females, $p=0.041$. The median number of side effects in males (5.00) was lower than in females (7.00).

To determine which specific side effects contributed to the sex-difference in total side effects, side effects were individually investigated, using a Chi-squared analysis. There was a significant difference in the frequency of three side effects between sexes, namely tremor ($p=0.035$), constipation ($p=0.001$) and stuffy nose ($p=0.048$). Tremor and constipation were both more prevalent in females and the only individuals to report experiencing a congested nose were female.

Table 5.3: Descriptive statistics of the side effect frequency in patients stratified in terms of sex and ethnicity

		N	Mean	Standard Deviation	Median	Variance	Minimum	Maximum
Sex	Male	37	5.57	3.524	5.00	12.419	0	13
	Female	68	6.97	3.266	7.00	10.666	0	16
Ethnicity	Caucasian	60	5.90	3.373	6.00	11.380	0	13
	Mixed	38	7.13	3.481	7.50	12.117	1	16
	Ancestry							

The median number of side effects in Caucasian patients were 6.00 (Variance=11.380) and 7.50 (Variance =12.117) in Mixed Ancestry patients. The total side effect frequency did not differ significantly between these two groups, $p=0.081$, but since the p -value was near 0.05 the side effects were investigated individually. There was a significant difference in the frequency of polydipsia ($p=0.046$), constipation ($p=0.013$), indigestion ($p=0.001$), metallic taste ($p=0.041$) and blurred vision ($p=0.021$), which were all more frequently reported by patients of Mixed Ancestry. Figure 5.12 demonstrates the proportional difference in side effect frequency between Caucasians and patients of Mixed Ancestry. Patients from other ethnic groups were excluded from this analysis due to small sample size.

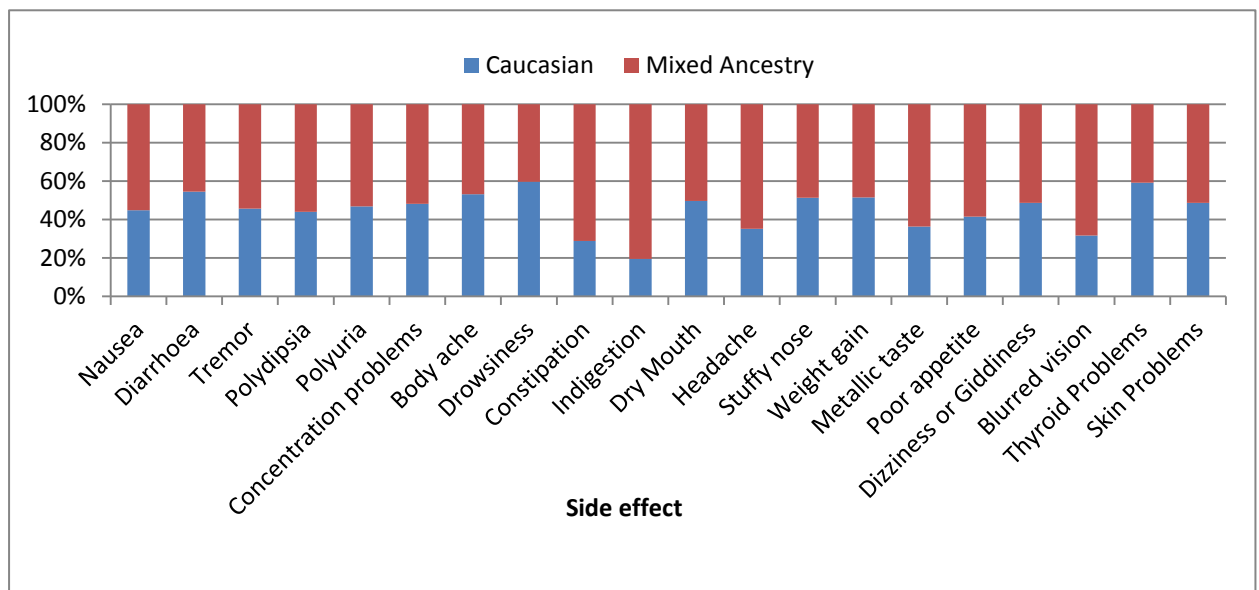


Figure 5.12: Proportional side effect frequency in Caucasian and Mixed Ancestry patients

5.5.3. The effect of other psychotropics on side effects

The influence of other psychotropics on the side effects experienced while on lithium was investigated, as several of the side effects of lithium are similar to those caused by other psychotropics. Data on other drugs used was not available for eleven patients as they could not remember their treatment regimen at the time of lithium use. Of the remaining patient cohort, 23.4% were treated with lithium alone. Nearly 77% of the cohort was therefore treated with between one and four other psychotropics. Of these patients 40.4% were also taking one or two anticonvulsants, 24.5% typical antipsychotics, 34.0% atypical antipsychotics and 26.6% were taking one or two antidepressants.

The group on lithium monotherapy was compared to the group of patients taking additional psychotropics. The frequency of side effects was normally distributed for the groups on lithium monotherapy and those taking other additional psychotropics, as assessed by Shapiro-Wilk's test ($p > 0.05$). A boxplot of this data is given in Figure 5.13.

Descriptive statistics of the side effect frequency in patients on lithium monotherapy and those using other additional psychotropics is given in Table 5.4. The mean number of side effects in the lithium monotherapy group was 6.86 (± 3.705) and 6.10 (± 3.188) in the group taking other psychotropics. There was no statistically significant difference in the side effects frequency in the two groups of patients, $p=0.349$.

The individual types of psychotropics were further investigated for association with the presence or absence of specific side effects. Nausea was negatively associated with the use of anticonvulsants ($p=0.032$) and memory and concentration problems were positively associated with the use of antipsychotics ($p=0.042$). Nausea appeared to be lower in patients taking anticonvulsants (6.7%) than in patients not being treated with anticonvulsants (22.5%).

Table 5.4: Descriptive statistics of the side effect frequency in patients on lithium monotherapy and patients using other additional psychotropics

	N	Mean	Std. Deviation	95% Confidence Interval for Mean		Minimum	Maximum
				Lower Bound	Upper Bound		
Lithium Monotherapy	21	6.86	3.705	5.17	8.54	1	12
Other Psychotropics	72	6.10	3.118	5.36	6.83	0	13

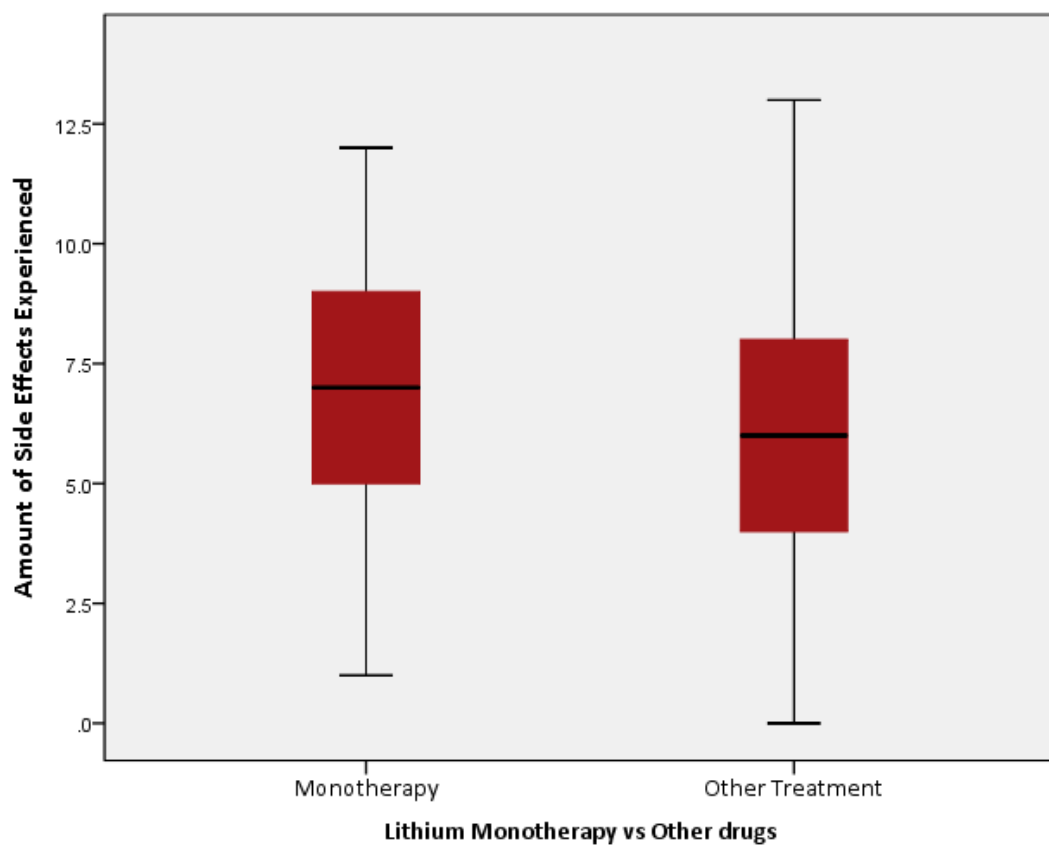


Figure 5.13: A boxplot of the side effects frequency in patients on lithium monotherapy versus patients taking other additional psychotropics.

5.6. Genotype-phenotype associations

A Kruskal-Wallis test was performed to determine if there were differences in the total number of side effects experienced between the different genotype groups of every SNP. There was no significant difference between the total number of side effects experienced and any of the SNP genotypes.

Next, a Chi-squared test for association was conducted to determine whether there is an association between genotypes of the ten candidate SNPs and the presence or absence of a particular side effect while on lithium treatment. Due to the small sample size, a genotypic genetic model could not be tested, as the majority of tests would have had one or more expected values smaller than five. Chi-squared tests for association were therefore conducted by grouping heterozygotes and minor allele homozygotes together. In cases where one or more expected frequencies were less than five, the Fisher's exact test was used. The results of the Chi-squared tests for association in the whole cohort are indicated in Table 5.5.

At the $p < 0.05$ level, there was an overall significant association between nausea and rs10138227 in *AKT1* ($p = 0.045$), with nausea more prevalent in homozygotes of the major allele (G). Polydipsia was associated with rs2279525 in *PPARGC1A* ($p = 0.034$) and more major allele (A allele) homozygotes experienced polydipsia.

Memory and concentration problems were associated with rs6438552 in *GSK3B* ($p = 0.028$), with the proportion of individuals experiencing memory and concentration problems higher in major C allele homozygotes. Weight gain was overall associated with rs1130233 ($p = 0.022$) in *AKT1*, with a larger proportion of patients with the GA or AA genotype gaining weight.

Body ache ($p = 0.030$), as well as skin problems ($p = 0.005$) were associated with rs8192678 in *PPARGC1A*. Skin problems were more likely to occur in individuals homozygous for the major G allele, while body ache was more prevalent in carriers of the minor A allele. Lastly, rs4302506 in *GRIA2* was associated with indigestion ($p = 0.012$), and indigestion was more frequently reported by carriers of the minor T allele. Bar plots of these associations is given in Figure 5.14.

Table 5.5: Chi-squared test for association of SNPs on presence or absence of side effects in complete cohort. Italicized results indicate that the Fisher's exact test was used.

Side Effect	<i>GSK3B</i>			<i>AKT1</i>		<i>ARRB2</i>		<i>GRIA2</i>	<i>PPARGC1A</i>	
	rs334558	rs6438552	rs3755557	rs1130233	rs10138227	rs34230287	rs1045280	rs4302506	rs8192678	rs2279525
Nausea	0.732	0.488	0.732	0.072	0.045	0.054	0.232	0.143	0.650	0.147
Diarrhoea	0.623	0.578	0.099	0.168	<i>0.552</i>	<i>0.111</i>	0.335	0.124	0.817	0.053
Tremor	0.796	0.772	0.185	0.904	0.450	0.959	0.174	0.541	0.211	0.551
Polydipsia	0.590	0.794	0.620	0.988	0.766	0.864	0.945	0.949	0.715	0.034
Polyuria	0.482	0.862	0.599	0.218	0.304	0.940	0.251	0.793	0.680	0.801
Concentration problems	0.089	0.028	0.055	0.401	0.297	0.269	0.081	0.159	0.784	0.551
Body ache	<i>1.000</i>	<i>0.763</i>	<i>0.771</i>	0.090	<i>0.343</i>	<i>0.518</i>	0.051	0.161	0.030	0.425
Drowsiness	0.813	0.956	0.568	0.266	0.881	0.899	0.639	0.222	0.227	0.780
Constipation	0.303	0.181	0.507	0.866	0.366	0.589	0.606	0.304	0.892	0.335
Indigestion	0.726	0.560	0.201	0.627	<i>1.000</i>	<i>1.000</i>	0.750	0.012	0.828	0.131
Dry Mouth	0.405	0.128	0.093	0.712	0.736	0.845	0.214	0.428	0.763	0.795
Headache	0.726	1.000	0.900	0.561	<i>0.549</i>	<i>0.645</i>	0.054	0.849	1.000	0.757
Stuffy nose	<i>1.000</i>	<i>1.000</i>	<i>1.000</i>	<i>1.000</i>	<i>0.425</i>	<i>0.675</i>	<i>0.290</i>	<i>1.000</i>	<i>0.202</i>	<i>1.000</i>
Weight gain	0.863	0.872	0.511	0.022	0.331	0.753	0.059	0.831	0.353	0.233
Metallic taste	0.612	0.678	0.834	0.089	0.577	0.096	0.196	0.659	0.929	0.297
Poor appetite	0.825	0.560	0.483	0.627	<i>1.000</i>	<i>0.759</i>	0.750	0.072	0.589	0.199
Dizziness or Giddiness	0.615	0.804	0.360	0.394	0.874	0.671	0.491	0.801	0.977	0.668
Blurred vision	0.574	0.799	0.667	0.507	0.446	1.000	0.294	0.261	0.637	0.875
Thyroid Problems	<i>0.763</i>	<i>0.753</i>	<i>0.754</i>	0.162	<i>0.509</i>	<i>0.295</i>	0.553	0.946	0.287	1.000
Skin Problems	0.052	0.094	0.066	0.528	0.797	0.589	0.070	0.705	0.005	0.526

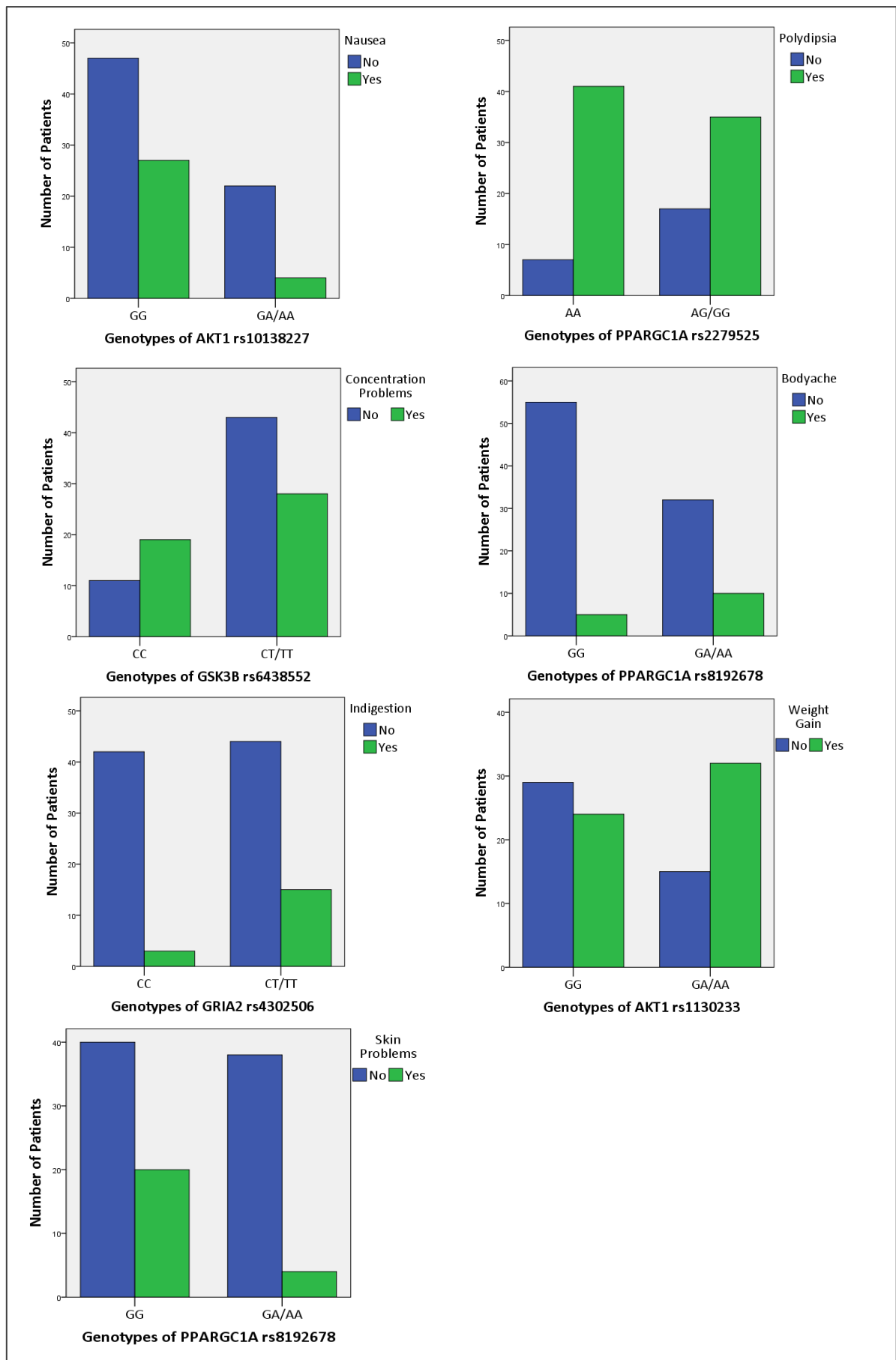


Figure 5.14: Bar plots of SNPs with significant association ($p < 0.05$) with presence of a side effect

5.6.1. Logistic regression modelling

Significant results were further assessed using backward stepwise logistic regression to determine whether the genetic associations remained significant after potential covariates (sex, age, ethnicity and the use of other psychotropics) were taken into account. In the case of genetic variables the major allele was used as the reference group to determine ORs. The result of the logistic regression analysis is given in Table 5.6. Variables that remained in the final step of the backward stepwise regression, with the respective p-values, OR and 95% CI for the ORs are given in the table.

The genetic variants that were further investigated were all included in the final step of the logistic regression models for the respective side effects. Only three genetic variants were statistically significant predictors on their own, rs6438552 as a predictor for memory and concentration problems ($p=0.034$), rs1130233 for weight gain ($p=0.034$) and rs8192678 for skin problems ($p=0.033$).

ORs indicated that carriers of the minor allele of rs6438552 were less likely to experience memory and concentration problems than individuals homozygous for the major allele (OR=0.309, 95% CI=0.104-0.916). The use of antipsychotics was also predictive of memory and concentration problems and individuals treated with antipsychotics in addition to lithium were 2.64 times (95% CI=1.037-6.722, $p=0.042$) more likely to experience these cognitive problems.

Carriers of the minor allele of rs1130233 in *AKT1* were 2.727 times more likely to experience weight gain than individuals homozygous for the major allele (95% CI=1.079-6.887). Antidepressants also remained as a covariate in the final step of the model, however, it was not a significant predictor for weight gain on its own ($p=0.065$). Individuals homozygous for the major allele of rs8192678 in *PPARGC1A* were 4.27 more likely to experience skin problems, with carriers of the minor allele being protected against skin problems (OR=0.234, 95% CI=0.062-0.891).

The other genetic variants were not significant predictors on their own. Anticonvulsants and the variant rs10138227 were predictors of nausea. Of the two predictor variants, only anticonvulsants were statistically significant and not rs10138227. Individuals treated with anticonvulsants were 3.37 times (OR=0.297, 95% CI 0.095-0.922, $p=0.034$) less likely to experience nausea than patients not taking anticonvulsants.

Both sex and ethnicity remained, together with rs4302506 in *GRIA2*, as covariates in the final step of the logistic regression model for indigestion. Only ethnicity was a significant predictor of indigestion on its own, with individuals of Mixed Ancestry being 7.163 times more likely to experience indigestion than Caucasians (95% CI=1.748-29.351). In the logistic regression model of polydipsia, age was a significant predictor ($p=0.027$, OR=0.939, 95% CI=0.888-0.993). Although not a statistically significant predictor, only rs8192678 remained as a covariate in the logistic regression model of body ache. OR indicated that carriers of the minor allele were 3.333 times more likely to experience body ache than major allele homozygotes (95% CI=0.916-12.128).

Table 5.6: Result of backward stepwise logistic regression analysis

Side effect	Covariates	p-value	Odds*	95% CI**	
			Ratio	Upper	Lower
Memory and Concentration	Antipsychotics	0.042	2.641	1.037	6.722
	rs6438552	0.034	0.309	0.104	0.916
Weight gain	Antidepressants	0.065	0.379	0.135	1.062
	rs1130233	0.034	2.727	1.079	6.887
Nausea	Anticonvulsants	0.036	0.297	0.095	0.922
	rs10138227	0.083	0.301	0.077	1.171
Indigestion	Ethnicity	0.006	7.163	1.748	29.351
	Sex	0.117	0.268	0.052	1.394
	rs4302506	0.081	3.614	0.855	15.273
Body ache	rs8192678	0.068	3.333	0.916	12.128
Skin problems	rs8192678	0.033	0.234	0.062	0.891
Polydipsia	Age	0.027	0.939	0.888	0.993
	rs2279525	0.077	0.351	0.110	1.120

*Major allele of SNP is reference group for each Odds Ratio

**95% CI – 95% confidence interval for the Odds Ratio of each variable

Chapter 6. Discussion

BPD is a severe, debilitating psychiatric illness that affects millions of individuals worldwide. There is no single cure or guaranteed treatment of BPD and it cannot be disputed that improved treatments are needed. Evidence-based drug development for BPD and other psychiatric disorders are limited as the aetiology of these diseases is not fully understood and the exact cause of the disease can therefore not be treated.

Several drugs are available to manage the symptoms of BPD, but due to disease heterogeneity and pharmacogenetic factors, treatment response varies significantly from patient to patient. The mood stabiliser, lithium, is the first-line treatment for BPD and many patients have an excellent response to lithium, tolerate it well, and are stabilized for years, while others do not. Response to lithium appears to have a strong genetic component and polymorphisms in several genes (amongst others *PLCG1*, *BDNF*, *FYN*, *5'-HTTLPR*, *INPP1* and *GSK3B*) have been associated with lithium response in some but not all studies. As there are no good predictors of response, determining the most effective treatment for a patient is conducted through a trial-and-error basis that can last months, prolonging patient suffering.

One of the main factors hindering the effectiveness of lithium in clinical practice is treatment non-adherence. In BPD patients, the rates of non-adherence range from 10 to 60% (Pompili et al., 2009). Patients often discontinue lithium treatment due to ADRs, such as weight gain and cognitive problems (Johnson et al., 2007), even if their mood symptoms are effectively managed with lithium. If the occurrence of side effects could be predicted prior to treatment initiation, alternative treatment could be considered or side effects could be managed before they occur.

In spite of association of ADRs with lithium dose and lithium serum levels, there are no accurate predictors of these side effects in lithium-treated patients. In the present pilot study, it was investigated whether certain genetic markers could be used to predict whether a patient would experience side effects while on lithium treatment. To the investigator's knowledge, no previous studies of the influence of genetic factors on lithium-induced ADRs have been reported, except for a recent study that reported an association of a SNP in *GSK3B* with renal concentrating ability (Rybakowski et al., 2013b).

A pilot study can be defined as an “investigation designed to test the feasibility of methods and procedures for later use on a large scale or to search for possible effects and associations that may be worth following up in a subsequent larger study” (Everitt, 2006). As this was an exploratory pilot study, the cohort size was small and the power to detect significant association was therefore low.

Correction for multiple testing was not conducted as interesting results for further research (as found in this study) would not have been detected. Multiple testing refers to the repeated use of a statistical test when multiple genes and interactions are considered. When considering a specific null hypothesis, the risk of type I errors (false positive results) increases with multiple testing. In exploratory studies, such as this study, however, there is no pre-specified key hypothesis that is tested and correction for multiple testing is not strictly required (Bender and Lange, 2001). Any significant results from this study are merely exploratory and to confirm these results the corresponding specific hypotheses have to be tested in further confirmatory studies.

6.1. Allele and genotype frequencies of SNPs

The majority of individuals in this study were part of two population groups, Caucasians and Mixed Ancestry. The genotype frequencies of SNPs did not differ significantly between these two groups, except for one SNP, rs334558, in *GSK3B*. The Mixed Ancestry (or Coloured) population of South Africa is a highly admixed, but discrete ethnic group that arose from indigenous Khoisan, tribal Bantu-speaking populations, European settlers, and slaves' descendants from south Asia (Quintana-Murci et al., 2010).

The allele frequencies in the Caucasian and Mixed Ancestry populations in the current study were compared to allele frequencies of Caucasians (CEU) included in the HapMap study (Table 6.1). There was a significant difference in allele frequencies of three SNPs (rs6438552, rs34230287 and rs8192678) between Caucasians and the HapMap CEU population. Interestingly, the allele frequencies of only one SNP, rs6438552 in *GSK3B*, differed significantly between the Mixed Ancestry group and the HapMap CEU population.

SNPs in *GSK3B* have previously been associated with BPD and the difference in allele frequency may therefore be due to only cases, and no healthy individuals, being included in this study. In a large meta-analysis the *GSK3B* variant, rs334558, was nominally associated with BPD (Seifuddin et al., 2012), however, no association was found with BPD I in our cohort in a previous study (Dalvie, unpublished data, 2011).

Even though these differences in allele frequencies should be further investigated in larger control populations, it highlights the importance of the study of population genetic diversity, as large differences may exist between population groups that may be assumed to be ethnically similar. This is important to take into account especially for association studies where population stratification can lead to false positive results.

According to the r^2 measure, no tight LD was observed between any of the SNPs and no haplotypes could be inferred. If the D' value is taken in to account, however, the D' value of 1.000 indicated that the three SNPs in *GSK3B* (rs334558, rs3755557 and rs6438552) are in complete LD in Caucasians and in tight LD in the Mixed Ancestry population. This is consistent with previous studies that found rs334558 and rs6438552 to be in LD (Kwok et al., 2005). The South African Caucasian population, especially the Afrikaner subset, is also known to be relatively homogenous and a high level of LD is observed (Hall et al., 2002).

Table 6.1: Comparison of allele frequencies in the study cohort with Caucasian HapMap (CEU) populations

Gene	SNP	Minor Allele	Study Cohort		HapMap CEU (n=113)	Comparison (p-value)	
			Caucasian (n=60)	Mixed Ancestry (n=38)		Caucasian	Mixed Ancestry
<i>GSK3B</i>	rs334558	T	0.38	0.58	N/A	N/A	N/A
	rs6438552	T	0.42	0.5	0.67	0.0001	0.009
	rs3755557	T	0.17	0.14	0.18	0.783	0.404
<i>AKT1</i>	rs1130233	A	0.18	0.32	N/A	N/A	N/A
	rs10138227	A	0.18	0.17	0.14	0.309	0.533
<i>ARRB2</i>	rs34230287	T	0.09	0.17	0.21	0.007	0.504
	rs1045280	C	0.31	0.22	0.32	0.924	0.119
<i>GRIA2</i>	rs4302506	T	0.41	0.30	0.36	0.361	0.376
<i>PPARGC1A</i>	rs8192678	A	0.18	0.29	0.35	0.001	0.337
	rs2279525	G	0.34	0.31	0.28	0.247	0.537

N/A: not available

6.2. Side effects

This study investigated the prevalence and severity of twenty of the most common side effects associated with lithium treatment. Further, the role of demographics, dose, serum lithium levels and the use of other psychotropics on side effect prevalence and severity, was investigated.

Almost all (97.2%) of the cohort reported experiencing at least one side effect, with only three patients (2.8%) reporting no observable side effects while on lithium treatment. Nephrogenic symptoms were the most commonly experienced side effects in our cohort. Polydipsia was reported by 75% of the patients, a dry mouth by 71% and polyuria by 60%. This is consistent with other studies that found approximately 70% of their cohort to experience increased thirst and urination (Vestergaard et al., 1980). Although not measured in this study, it has been found that almost all patients have an irreversibly reduced maximum urinary concentration capacity after 15 years on lithium treatment (Bendz et al., 1996; Bendz et al., 2009). A meta-analysis by McKnight and colleagues reported glomerular filtration rates to be reduced by – 6.22 ml/min and the urinary concentrating ability by 15% of normal maximum (McKnight et al., 2012). Three patients in this cohort had to discontinue lithium treatment due to kidney problems and at least one of these patients is known to have permanent renal damage. In a recent meta-analysis the absolute risk of developing renal failure was reported to be only 0.5% (McKnight et al., 2012). Due to the seriousness of renal failure, however, serum creatinine levels should be routinely tested and lithium-treatment discontinued before any permanent renal damage can occur.

Gastrointestinal side effects, such as nausea, vomiting, and diarrhoea are common complaints in lithium-treated patients and previous studies report that up to 50% of patients may experience one or more of these complaints (Bowden et al., 2000). In this study, 31% of patients reported experiencing nausea, 25% constipation, 18% diarrhoea, and 17% complained of indigestion. These side effects are often transitory, but may be the first sign of lithium toxicity and therefore needs to be monitored (Macritchie and Young, 2004). The frequency of constipation was significantly higher in females than in males. Constipation is more prevalent in females than in males in the general population, which may explain the difference in prevalence in this study, which is also consistent with other studies (McCrea et al., 2009).

Tremor was experienced by 54% of patients when lithium treatment was first started. Individual studies on tremor show variability of 4% to 65%, with the average frequency of tremor estimated to be about 27% of patients treated with lithium (Gelenberg and Jefferson, 1995). In this study tremor was experienced significantly more by females (62.7%) than in males (40.5%). This differs from previous studies, which found the frequency of tremor to be equal in males and females (Vestergaard et al., 1988) or more common in males (54%) than in females (33%) (Vestergaard et al., 1980).

Endocrine side effects of lithium, such as thyroid dysfunction can have dire consequences when not properly managed. In this study, the frequency of thyroid problems was 14%, although the severity of the specific thyroid problems was not measured. The prevalence of hypothyroidism in the general population ranges from 2-3% (Flynn et al., 2004). In lithium-treated patients overt and subclinical hypothyroidism has been reported to be 8-19% and 23%, respectively (Kleiner et al., 1999). In the Chinese population, lithium-treated patients also experienced a higher rate of hypothyroidism (28-32%) than BPD patients not treated with lithium (6.3-10.8%) (Zhang et al., 2006). A recent meta-analysis, reported hypothyroidism to be more common in lithium-treated patients than controls, and TSH was increased by 4.00 iU/mL (95% CI 3.90–4.10, $p < 0.0001$) on average (McKnight et al., 2012).

Nearly half (46%) of the present cohort reported experiencing memory and concentration problems, i.e. cognitive problems, while on lithium treatment. Poorer cognitive performance is a common impairment in BPD patients and studies have shown BPD patients to have impaired functioning across a range of cognitive domains, independent of treatment (Martinez-Aran et al., 2005). Lithium therapy has been associated with minor negative effects on cognition (Wingo et al., 2009).

Weight gain is a frequent and significant problem in patients with BPD, with or without the influence of medication. In this study, weight gain was reported by 55% of patients, compared to 25-50% of patients in previous studies (Keck Jr and McElroy, 2002; Goodwin and Jamison, 2007). A prospective study conducted over seven years, reported weight gain to occur during the first two years of lithium treatment, after which weight stabilised (Vestergaard et al., 1988). Weight gain may be explained by fluid retention, increased appetite, lithium-related subclinical hypothyroidism or a combination of these and other biological or genetic factors (Torrent et al., 2008).

Dermatological side effects of lithium treatment can be very distressing for patients and negatively affect treatment adherence. In controlled trials, 3.4–45% of patients treated with lithium developed dermatological side effects, with acne and psoriasis the most common (Yeung and Chan, 2004). A quarter of this cohort reported experiencing a dermatological side effect, with the most common complaints being acne and unusually dry skin.

Headaches and body aches were experienced by 17% and 15% of the cohort, respectively. This is consistent with other studies, in which headaches were reported by 13.2-23.9% of lithium-treated patients (El-Mallakh et al., 2012). In the same study the combined percentage of extremity pain, musculoskeletal stiffness and back pain was 11.9-18.1% (El-Mallakh et al., 2012).

The experience of a metallic taste (dysgeusia) was reported by 34% of patients. This may be due to a reduction in salivary flow, which concentrates electrolytes in the saliva, resulting in a metallic taste. Other side effects that were reported in more than 20% of patients were drowsiness (31%), blurred vision (28%) and dizziness (21%).

Lithium dosage did not have a significant effect on the total prevalence of side effects. This may be due to the fact that serum lithium levels were routinely tested in these patients and dose was therefore stabilised within the therapeutic serum range. In previous studies, lithium serum level was associated with prevalence and severity of reported side effects (Wilting et al., 2009). In this study, there was no correlation between lithium serum levels and side effect prevalence. Due to incomplete patient folders and a lack of access to the records of some patients, the levels of only seventeen patients were known to the researcher, and accurate conclusions cannot be drawn.

Patient sex was associated with the total prevalence of side effects, with specifically tremor, constipation and a congested nose being significantly more prevalent in females than males. Patient ethnicity was also associated with the frequency of some side effects, with polydipsia, constipation, indigestion, metallic taste and blurred vision reported more frequently by patients of Mixed Ancestry. The difference in these side effects may be influenced by genetic differences between these population groups, but may also be influenced by cultural, socio-economic and other population-specific environmental factors.

The number or type of concomitant medications did not significantly affect the prevalence of side effects. This is contradictory to other studies that found that side effects increased with number of drugs (Serretti et al., 2013). Specific treatment type also did not have an association with individual side effects, except for two side effects.

Memory and concentration problems were significantly associated with treatment with antipsychotics ($p=0.042$), and nausea with the use of anticonvulsants ($p=0.036$). Memory and concentration problems have previously been reported to be associated with antipsychotics in the large STEP-BD study (Serretti et al., 2013).

6.3. Genotype-Phenotype associations

The main aim of this study was to determine whether variants in five selected candidate genes are risk factors for the development of side effects while treated with lithium. Associations were found between genetic variants in four of the five candidate genes and several side effects, including weight gain, skin and cognitive problems.

6.3.1. Associations with *AKT1*

Two side effects of lithium were associated with SNPs in *AKT1*. Weight gain was associated with rs1130233 in *AKT1*, and nausea was associated with rs10138227 in *AKT1*. The association of nausea, however, did not remain significant when the association of nausea with anticonvulsant use was taken into account.

AKT1 (or Protein Kinase B) is a serine/threonine-protein kinase involved in the regulation of several growth factors. AKT1 regulates glucose transporter 4 (GLUT4), GSK3, and tuberous sclerosis complex 2 (TSC2)-mTOR signalling pathways thereby stimulating glucose uptake (Welsh et al., 2005), glycogen synthesis (Cross et al., 1995) and protein synthesis (Ruggero and Sonenberg, 2005; Memmott and Dennis, 2009). Akt activation in skeletal muscles leads to muscle hypertrophy (Rommel et al., 2001) and reduced fat accumulation (Izumiya et al., 2008). In insulin-resistant tissues of obese mice AKT/mTOR activity is decreased (Shao et al., 2000) and GSK3B activity increased (Kaidanovich-Beilin and Woodgett, 2011). Akt1 transgene-induction in obese mice leads to muscle growth, weight loss and the improvement of metabolic disorders (Izumiya et al., 2008).

Lithium treatment leads to increased Akt expression in the striatum, frontal cortex, and hippocampus of rodents (Beaulieu et al., 2004) and concurrent increase in GSK3 phosphorylation and inhibition (Chalecka-Franaszek and Chuang, 1999), which would

normally not be associated with weight gain. Weight gain was more frequently reported by carriers of the *AKT1* rs1130233 minor A allele, in which expression of *AKT1* is significantly reduced when compared to major allele homozygotes (Tan et al., 2008). These individuals may be less susceptible to the effects of lithium on *AKT1* expression.

Higher levels of *AKT1* (in patients homozygous for the major allele) may therefore be protective against lithium-induced weight gain and modify a patient's likelihood of gaining weight while on lithium treatment.

6.3.2. Associations with *PPARGC1a*

The SNP rs8192678 in *PPARGC1a* was associated with body ache and skin problems and rs2279525 was associated with polydipsia. Skin problems were reported by 24% of subjects, and were associated with the G allele in the *PPARGC1a* SNP rs8192678. *PPARGC1a* encodes for a transcriptional co-activator, PGC-1 α , which increases the transcriptional activity of several nuclear receptors, including PPAR- α and PPAR- γ . Decreased activity of PPAR- α and PPAR- γ and of multiple genes regulated by these nuclear receptors, can be seen in several skin conditions, including atopic dermatitis (Plager et al., 2007) and psoriasis (Romanowska et al., 2010). PPARs have anti-inflammatory activity in the skin and modulate epidermal proliferation (Schmuth et al., 2008).

The A allele of rs8192678 in *PPARGC1a* is associated with reduced mRNA expression and lower levels of PGC-1 α (Ling et al., 2008). The expression of *PPARGC1a* is inhibited by lithium in human neuronal cell lines (Seelan et al., 2008) and to almost undetectable levels in brown preadipocytes during brown fat differentiation (Rodríguez de la Concepción et al., 2005). The inhibition of *PPARGC1a* by lithium may lead to decreased transcriptional activity of PPAR- α and PPAR- γ , and increased risk of inflammatory skin conditions or skin conditions associated with increased cell turnover.

Individuals carrying the A allele of rs8192678 were more likely to experience body ache, although in the logistic regression model of body ache, rs8192678 was not a significant predictor of this side effect. However, the association seen with Chi-squared analysis may also be mediated through PPARs. PPARs play an essential role in inflammation which may contribute to joint pain (Jhaveri et al., 2008). In addition, mice lacking PPAR- α have shown higher sensitivity to neuropathic pain (Ruiz-Medina et al., 2012) and PPAR- α agonists suppress pain behaviour in mice (LoVerme et al., 2006).

Although also not significant in the logistic regression model, the association of the 5'UTR variant rs2279525 in *PPARGC1α* with polydipsia is of interest due to the important role of this co-activator in the kidneys. PGC-1α is expressed at high levels in the kidneys where it is particularly important for its role in oxidative metabolism and mitochondrial biogenesis (Bjornholm and Zierath, 2005).

The repression of PGC-1α expression resulted in downregulation of important mitochondrial enzymes and the loss of and morphological changes to mitochondria (Czubryt et al., 2003). PGC-1α has been demonstrated to be essential for the maintenance of mitochondrial function in podocytes (Yuan et al., 2012). Podocytes are glomerular epithelial cells that are a critical component of the glomerular filtration barrier in the kidneys; and have been reportedly damaged in some patients with lithium-induced nephrotoxicity (Markowitz et al., 2000). In addition, PPAR agonists can affect glomerular and tubular cells to modify acute and chronic kidney injury (Ruan et al., 2008; Wang et al., 2010). Impairment of PPAR and PGC-1 α signalling has been reported in cisplatin-induced acute kidney injury (Portilla et al., 2002). It has further been ascertained that PPARs are significantly involved in a mouse model of X-linked nephrogenic diabetes insipidus (Schliebe et al., 2008).

6.3.3. Associations with *GSK3B*

Cognitive problems were reported by 45% of subjects, and of the *GSK3B* SNPs investigated, rs6438552 C allele homozygotes were 3 times more likely to report these than carriers of the T allele. This variant influences *GSK3B* alternative splicing; the T allele of rs6438552 results in greater levels of the *GSK3B* more active exon9+11 isoform and increased enzyme activity in human lymphocytes (Kwok et al., 2005). *GSK3B* inhibition has positive effects on neurogenesis (King et al., 2013) and is protective against inflammation and apoptosis (Song et al., 2002; Watcharasit et al., 2002). The rs6438552 T allele is associated with a poor response to lithium, measured by recurrence of episodes (Lin, 2012).

The effect of lithium on cognition in BPD patients has been repeatedly studied, but results remain contradictory, in particular because neurocognitive deficits are very common in BPD patients regardless of treatment (Balanzá-Martínez et al., 2010; Dias et al., 2012). Several studies have concluded that the cognitive abilities of lithium-treated BPD patients does not differ from medication-free patients, while others have found lithium to have a mild negative effect on attention, verbal learning and memory, and short-term memory (King et al., 2013). A meta-analysis on lithium and cognition concluded that lithium only has minor negative effects on cognition (Wingo et al., 2009).

In conditions with symptoms of cognitive impairments, the administration of GSK3 inhibitors has significantly enhanced cognitive abilities. In Alzheimer's disease (AD), for example, the upregulation of GSK3 is thought to be the link between β -amyloid and tau protein, and GSK3 upregulation has been used to model AD pathologies (Martinez et al., 2011).

Lithium treatment reduced neuropathology and cognitive deficits in these models through inhibition of GSK3 (Rockenstein et al., 2007). Studies of the effect of moderate dosages of lithium or other GSK3B inhibitors on cognition in healthy rodents or humans, however, indicated mild or no effects (King et al., 2013).

In BPD patients, the long-term administration of lithium and the less active C allele of the *GSK3B* promoter SNP, rs334558, have both been associated with increases in measures of axial diffusivity in several white matter tracts, which reflects the integrity of axons and myelin sheaths (Benedetti et al., 2012). The C allele of rs334558 was also associated with protective effects against grey matter loss in schizophrenia (Benedetti et al., 2010). Similarly, variation in grey matter volume in patients with MDD has been associated with rs6438552 (Inkster et al., 2009). On the other hand, GSK3 inhibition by lithium elicits NFAT/Fas mediated neuronal apoptosis, which may lead to, amongst others lithium-induced motor deficits (Gómez-Sintes and Lucas, 2010). In this study, a trend towards association ($p=0.089$) was seen between cognitive problems and rs334558 in *GSK3B*, but these problems were proportionally more common in individuals homozygous for the C allele and the C allele was therefore not protective as in previous studies (Benedetti et al., 2010).

In a recent study on SNPs involved in late-onset AD, the minor T allele of rs6438552 was associated with a protective effect against AD, and the CC genotype was twice as common in AD patients as in controls (Izzo et al., 2013). The protective effect of the T allele was, however, dependent on the Apolipoprotein E (APOE) status of the patients, indicating it to be a risk factor for APOE*4-positive carriers (Izzo et al., 2013).

Similarly, in a study of Parkinson's disease (PD) the effect of *GSK3B* polymorphisms were dependent on other risk factors. The transcriptionally more active TT haplotype of rs334558 and rs6438552 increased or decreased the risk for PD depending on the microtubule-associated protein Tau (*MAPT*) haplotype (Kwok et al., 2005). This may be owing to the differential phosphorylation of Tau by the different splice variants of *GSK3B* (Soutar et al., 2010).

The inhibition of GSK3B may, therefore, elicit positive or negative effects on cognition depending on several other factors. This has also been demonstrated in a rodent model, where the overexpression of GSK3B lead to spatial memory impairments (Hernández et al., 2002), and deficits in object recognition tasks that were reversed when GSK3B levels were restored to normal (Engel et al., 2006). Yet, in a *GSK3B* hypogene model, spatial memory was similar to wild type mice, but long-term memory formations were adversely affected (Kimura et al., 2008).

The results of this study may be important to consider in the proposed development of drugs that selectively inhibit *GSK3B* for both the treatment of mood disorders (Li et al., 2011) and AD (Mondragón-Rodríguez et al., 2012), as the response of a patient may be altered by the patient's genotype. Functional studies are also needed to further elucidate the differential role of splice variants of *GSK3B* (influenced by rs6438552), which may significantly alter the protein function and its interaction with other proteins, such as Tau and APOE (Soutar et al., 2010).

6.3.4. Associations with *GRIA2*

Glutamate receptor, ionotropic, AMPA 2 (*GRIA2*) is one of a family of glutamate receptors that are sensitive to AMPA, and function as ligand-activated cation channels. These channels are assembled from four related subunits, namely, GRIA1-4. *GRIA2* is essential for the channel to be permeable to calcium. *GRIA2* is down-regulated by chronic lithium treatment in human neuronal cell lines (Seelan et al., 2008) and chronic lithium treatment decreases the levels of GluR2 in hippocampal neurons (Du et al., 2008).

Although it did not remain significant in a logistic regression model, chi-squared analysis indicated a variant in *GRIA2*, rs4302506, to be associated with reported indigestion in patients. Indigestion is a common side effect of glutamate supplements, and we hypothesise that lithium-induced decrease of glutamate receptors in the gut may contribute to indigestion.

Glutamate is a multi-functional amino acid and in addition to its role as neurotransmitter, it plays a critical role in the stomach and intestine. Both ionotropic (e.g. *GRIA2*) and metabotropic glutamate receptors occur in the gut, where the presence of protein digestion is signalled in the gastrointestinal tract via glutamate (Burrin and Stoll, 2009). The intra-gastric glutamate stimulates the limbic system and hypothalamus, as well as gastric contractile activity (Sengupta et al., 2004).

Although not previously studied (to the author's knowledge), downregulation of glutamate receptors, such as GRIA2, may therefore contribute to indigestion. The variant rs4302506 is a synonymous coding variant and is not predicted to be harmful, but may be in LD with a functional variant.

6.4. Limitations and future directions

The current study was a pilot and due to the relatively small sample size there was not sufficient power to determine true association. In addition, no causal relationship can be determined from associations in this study. As side effects were subjectively reported, it is difficult to assess the true severity and how common side effects are in patients; and since data was collected retrospectively, the accuracy of the patient's memory also needs to be taken into account.

The majority of the cohort was telephonically contacted and often not on lithium treatment at the time of the interview, which may cause a form of recall bias. A possible cofounder may also be the two methods through which patients were contacted (telephonically and personally within the hospital). Some patients recruited from the hospital have not been on lithium treatment for as long a long period of time and their memory of side effects may therefore be more accurate (or more severe) than patients who have received treatment for a longer period of time. Data on serum lithium levels were also not always available, and since serum lithium levels have previously been associated with the prevalence and severity of ADRs, this data is important for future studies.

Future studies on lithium ADRs should be prospective rather than retrospective, as the severity of a side effect can be noted as it occurs, and the duration recorded. Access to objective laboratory measures on factors such as urine concentrating capacity and TSH levels, accurate records of weight changes, and physician observations on tremor would also be advantages of a prospective study. As the number of side effects has been linked to mood state in previous studies of side effects in BPD patients (Wilting et al., 2009; Serretti et al., 2013), mood state at the time of side effect reporting should also be evaluated.

The findings of this study should further be investigated in larger, better characterised cohorts to determine whether results can be replicated. As this was a pilot study, no correction for multiple testing was performed, which significantly increases the risk of type I errors. Results may also be significantly influenced by population stratification. Functional studies on the investigated variants are also necessary as the statistical significance of a genotype with a phenotype does not illustrate the biological mechanism for risk. Therefore, the underlying biology needs to be determined through functional analysis of the SNPs.

The associated SNP rs2279525, in *PPARGC1α*, is located in the 5'UTR and the SNP rs1130233, in *AKT1*, and rs4302506, in *GRIA2*, are synonymous SNPs and the impact on gene function is

unknown. Instead of affecting protein function or formation directly, the SNPs may affect how genes are regulated and expressed, or be in LD with a functional SNP. Understanding the function of the SNPs would help to elucidate the mechanism of lithium's interaction with or effect on these genes.

Future genetic studies on lithium response or lithium-induced ADRs may also consider taking a pathway-based approach, as opposed to a candidate gene approach. For a pharmacogenetic test to be useful in clinical practice, it must provide more information than can be obtained from clinical history. No pharmacogenetic finding on lithium response has managed to accomplish this and each genetic polymorphism reported, in this and other studies, only accounts for a small portion of the total variance. Evidence suggests that high-penetrance variants capable of explaining the full genetic variability are rare. It is, therefore, likely that (as with BPD) lithium response is polygenic and any single gene may have multiple polymorphic alleles. Multiple genes and multiple variants within these genes would therefore have to be examined to form the basis of a genetic test.

One way to approach this problem is to investigate candidate pathways instead of candidate genes. GWAS and genome-wide expression studies allow for the discovery of new genes not previously discovered, which can be analysed and organised with various bioinformatic tools to prioritise the multitude of interactions between genes. Candidate pathway approaches make the assumption that a phenotype such as response to lithium (including the development of ADRs) may arise from multiple, distinct elements of a common biological process. These variants (concentrated within a pathway) may have more robust effects than distantly related variants. Through epistasis, these related genes may interact to modify the effects of a specific variant. As lithium's mechanism of therapeutic action (and its off-target effects) are known to involve several complex and inter-related processes, a candidate pathway approach to its investigation may be more successful than traditional methods.

A further dimension of drug response that should be investigated is the possible effect of gene environment (GxE) interactions. Studies on MDD have indicated that environmental factors may explain some of unexplained heterogeneity in treatment response (Keers and Uher, 2012). In a study of 383 patients treated with the antidepressant escitalopram, the SS genotype of 5-HTTLPR was associated with poor response, but only when the patient reported experiencing one or more stressful life events (Keers et al., 2011). As environmental factors are known to play a role in the etiology of BPD, these same factors could also modulate response to amongst others, lithium treatment.

6.5. Conclusion

The current study showed that interindividual susceptibility to developing side effects on lithium treatment may be mediated by genetic factors. Surprisingly, concomitant treatment with other psychotropics was not significantly associated with lithium ADRs, except for associations between nausea (with anticonvulsants) and memory and concentration problems (with antipsychotics). Of particular interest, is the associations found in this study between weight gain and an *AKT1* variant, cognitive (memory and concentration) problems and a *GSK3B* variant and cutaneous problems and a SNP in *PPARGC1α*. These side effects can negatively contribute to patient adherence and understanding some of the genetic factors contributing to interindividual susceptibility may aid in better management of patients with BPD. This study further contributes to our understanding of lithium's mechanism of action. This is a pilot study and cannot have an immediate impact on treatment prediction. It, however, lays the foundation for future studies, the results of which may be important in not only the prediction of response to lithium, but also in the development of new pharmacotherapies for BPD through target-based drug discovery.

Electronic Resources

National Centre for Biotechnology Information (NCBI):

<http://www.ncbi.nlm.nih.gov/>

NCBI PrimerBLAST program:

<http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi>

OligoAnalyzer (v3.1 2011 Integrated DNA technologies):

<http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx>

OligoCalculator:

<http://www.pitt.edu/~rsup/OligoCalc.html>

Ensembl:

<http://www.ensembl.org/index.html>

Primer3 primer selection program:

<http://frodo.wi.mit.edu/primer3/>

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Appendix A: Ethics Clearance letter from the University of Cape Town

UNIVERSITY OF CAPE TOWN



Faculty of Health Sciences
Human Research Ethics Committee
Room E52-24 Groote Schuur Hospital Old Main Building
Observatory 7925
Telephone [021] 406 6338 • Facsimile [021] 406 6411
e-mail: lamees.emjedi@uct.ac.za

06 December 2012

HREC REF: 431/2012

Prof R Ramesar
Human Genetics
Medical School

Dear Prof Ramesar

PROJECT TITLE: THE PHARMACOGENETICS OF LITHIUM TREATMENT IN A SOUTH AFRICAN COHORT WITH BIPOLAR DISORDER

Thank you for your responses to the matters raised by the Faculty of Health Sciences Human Research Ethics Committee.

It is a pleasure to inform you that the Ethics Committee has **formally approved** the above-mentioned study.

Approval is granted until 15 December 2013.

We approve Ms Reinette Weideman as the Masters student on this project.

Please submit to the HREC a Progress Report Form if the study continues beyond the approval period. Please submit a Closure Report Form on completion of the study. (Forms can be found on our website: <http://www.health.uct.ac.za/research/humanethics/forms/>)

Please note that the ongoing ethical conduct of the study remains the responsibility of the principal investigator.

Please quote the REC. REF in all your correspondence.

Yours sincerely

PROFESSOR MARC BLOCKMAN
CHAIRPERSON, FHS human research ethics committee

Federal Wide Assurance Number: FWA00001637.
Institutional Review Board (IRB) number: IRB00001938

Lemjedi

Appendix B: Patient Consent Form



REQUEST FOR MOLECULAR STUDIES (DNA)



Molecular Laboratory
Division of Human Genetics
IDMM, LEVEL 3
UCT Medical School, Observatory 7925

Tel: (021) 406 6425 Fax: (021) 406 6826

Blood should be drawn in 2 plastic EDTA Tubes
(Purple top) +/- 10ml each using a yellow barrel
Each tube should be inverted to mix and should be
clearly labelled with the patient's name and DOB
Keep blood in fridge at 4°C until able to send to laboratory

Please **DO NOT** send specimens on ice or frozen.

Please fill in all the information requested.

Surname: _____ First Name(s): _____

New Family: Yes ☐ No ☐ (If no, please fill in family name) Family name: _____

Medical Aid: _____ Medical Aid No: _____

Sex: M ☐ F ☐ Date of Birth: Year: _____ Month: _____ Day: _____

Number of children: _____

Ethnic Origin: (please indicate ancestry of both your mother and father) _____

Contact Address: _____ Town: _____ Fax: _____
Tel: _____

Referring Doctor/Sister: _____ Town: _____ Fax: _____
Tel: _____

Hospital or Address: _____ Town: _____ Fax: _____
Tel: _____

Reason for Referral (Clinical diagnosis):

Affected ☐ At Risk ☐ Carrier ☐ Spouse ☐ Query ☐ Unaffected ☐

Becker Muscular Dys. ☐ Duchenne Muscular Dys. ☐ Colonic Carcinoma ☐

Fragile-X Syndrome ☐ Bipolar Disorder ☐ Huntington Disease ☐

Retinitis Pigmentosa ☐ Spinocerebellar Ataxia ☐ Waardenburg Syndrome ☐

Additional disorders (suspected or previously tested): _____

Additional family history: _____

Clinical Details:

Physical disability ☐ Mental retardation ☐ Deafness ☐ Impaired vision ☐ Night blindness ☐

Other: _____

Have samples from this patient been sent to a DNA lab before? (DELETE WHERE NOT APPLICABLE) YES / NO / Don't Know

If Yes, where: _____

For Laboratory use only:

DNA number: _____ Vol. Blood: _____ (ml) UNW: _____

Date Received: Year: _____ Month: _____ Day: _____ Computer Index No: _____

CONSENT FOR DNA ANALYSIS AND STORAGE

- I, _____, request that an attempt be made using genetic material to assess the probability that I / my child / my unborn child (DELETE WHERE NOT APPLICABLE) might have inherited a disease-causing mutation in the gene for _____.
- I understand that the genetic material for analysis is to be obtained from: blood cells/skin sample/other (specify) (DELETE WHERE NOT APPLICABLE): _____.
- I request that no portion of the sample be stored for later use. ☐ (MARK IF APPLICABLE) Or I request that a portion of the sample be stored indefinitely for (DELETE WHERE NOT APPLICABLE):
(a) possible re-analysis
(b) analysis for the benefit of members of my immediate family
(c) research purposes subject to the approval of the University of Cape Town Research Ethics Committee, provided that any information from such research will remain confidential.
- The results of the analysis carried out on this sample of stored biological material will be made known to me via my doctor, in accordance with the relevant protocol, if and when available.
In addition, I authorise that they may be made known to: (DELETE WHERE NOT APPLICABLE):
other doctors involved in my care: _____
the following family members: _____
other: _____
- I authorise / do not authorise my doctor(s) (DELETE WHERE NOT APPLICABLE) to provide relevant clinical details to the Division of Human Genetics, UCT.
- I have been informed that:
(a) there are risks and benefits associated with genetic analysis and storage of biological material and these have been explained to me.
(b) the analysis procedure is specific to the genetic condition mentioned above and cannot determine the complete genetic makeup of an individual.
(c) the genetics laboratory is under an obligation to respect medical confidentiality.
(d) genetic analysis may not be informative for some families or family members.
(e) even under the best conditions, current technology of this type is not perfect and could lead to incorrect results.
(f) where biological material is used for research purposes, there may be no direct benefit to me.
- I understand that I may withdraw my consent for any aspect of the above at any time without this affecting any future medical care.
- ALL OF THE ABOVE HAS BEEN EXPLAINED TO ME IN A LANGUAGE THAT I UNDERSTAND AND MY QUESTIONS ANSWERED BY.

DATE: _____

Patient signature: _____ Witnessed consent: _____

NOTE - PLEASE INSERT A FAMILY PEDIGREE DRAWING ON THE REVERSE OF THIS FORM

Appendix C: Side effect Questionnaire

Lithium Side Effect Questionnaire

Contact details:

Name:

Surname:

Cell number:

Email address:

Lithium questions:

Date you started on lithium treatment?

How would you rate your compliance to lithium? Do you take your medication:

75-100% of the time ☐ 51-75% of the time ☐ 26-50% of the time ☐ Less than 25% of the time ☐

What was/is the usual daily dose of lithium that you're on?

Do you know your current lithium serum level?

Can you remember whether you are/were on any other medications while taking lithium? If yes, what?

Please score each of the following symptoms in terms of severity according to nil (=0), mild (=1), moderate (=2) and severe (=3)

In first few weeks after starting lithium treatment did you suffer from?

Nausea 0 ☐ 1 ☐ 2 ☐ 3 ☐

Diarrhoea 0 ☐ 1 ☐ 2 ☐ 3 ☐

Tremor 0 ☐ 1 ☐ 2 ☐ 3 ☐

Excessive Thirst 0 ☐ 1 ☐ 2 ☐ 3 ☐

Passing urine too often 0 ☐ 1 ☐ 2 ☐ 3 ☐

While being on lithium treatment did you suffer from?

Memory/concentration problems 0 ☐ 1 ☐ 2 ☐ 3 ☐

Body ache 0 ☐ 1 ☐ 2 ☐ 3 ☐

Drowsiness 0 ☐ 1 ☐ 2 ☐ 3 ☐

Constipation 0 ☐ 1 ☐ 2 ☐ 3 ☐

Indigestion 0 ☐ 1 ☐ 2 ☐ 3 ☐

Dry mouth 0 ☐ 1 ☐ 2 ☐ 3 ☐

Headache 0 ☐ 1 ☐ 2 ☐ 3 ☐

Stuffy nose 0 ☐ 1 ☐ 2 ☐ 3 ☐

Weight gain 0 ☐ 1 ☐ 2 ☐ 3 ☐

Metallic Taste 0 ☐ 1 ☐ 2 ☐ 3 ☐

Poor appetite 0 ☐ 1 ☐ 2 ☐ 3 ☐

Dizziness or giddiness 0 ☐ 1 ☐ 2 ☐ 3 ☐

Blurred vision 0 ☐ 1 ☐ 2 ☐ 3 ☐

Thyroid problems 0 ☐ 1 ☐ 2 ☐ 3 ☐

Skin problems 0 ☐ 1 ☐ 2 ☐ 3 ☐

Specify which skin problems:

Did the doctor advise you to stop lithium treatment? If yes what was the reason?

Any additional comments regarding your treatment with lithium:

Appendix D: DNA isolation from blood

Salting-out method adapted from Gustafson et al. 1988 (Gustafson et al., 1987)

1. Obtain buffy coat by centrifuging the sample at 2 000rpm for 10mins
2. Remove 500 µl of buffy and plasma
3. Add 900 µl red blood cell (RBC) lysis solution and incubate at 37°C for 1 hour
4. Centrifuge at 2 500 rpm for 10mins
5. Remove supernatant and add 1 000 µl RBC lysis solution to pellet
6. Centrifuge 2 500 rpm/ 10 mins
7. Decant supernatant and resuspend the pellet in residual RBC lysis solution
8. Add 300 µl Cell Lysis Solution, 10 µl of 20% SDS and 2 µl of 20mg/ml Protease K
9. Vortex well
10. Incubate overnight or at room temperature until the solution clears (~2hours)
11. Add 200 µl of saturated 6M NaCl and vortex vigorously
12. Incubate at 4°C for 5 mins
13. Pellet cellular debris at 2 500 rpm for 15 mins
14. Remove 500 µl of supernatant and add to a fresh tube containing 900 µl ice-cold 100% EtOH
15. Mix by inversion (50X) and spin at 10 000rpm for 2mins
16. Decant the solution and add 400 µl ice-cold 70 % EtOH
17. Resuspend pellet by vortex
18. Recover DNA by centrifuging at 10 000 rpm for 2 mins
19. Pour off EtOH and allow to air dry for 2 hours
20. Resuspend in 100 µl 1X TE for at least 2 days

DNA quality determination

DNA integrity was determined by electrophoresis through a 1% agarose gel. Bands, on the gel, which appear thick and bright were indicative of non-degraded, good quality DNA. DNA fragments of lower molecular weight, in which double-stranded breaks have occurred, migrated further than fragments of higher molecular weight. DNA which appeared as a smear on the gel indicated that the DNA samples were degraded. An example of a DNA integrity gel is given in Figure A.

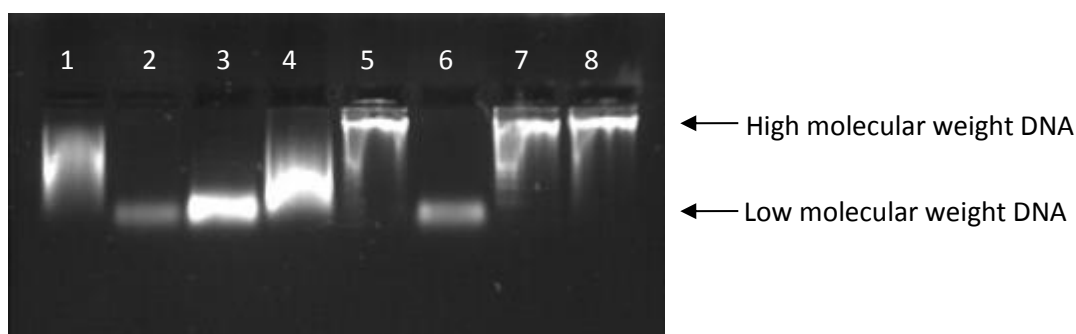


Figure A: Agarose gel electrophoresis of DNA isolated from peripheral blood lymphocytes. Low and high molecular weight DNA samples are indicated with arrows. A smear on the gel indicates that the DNA is slightly degraded, as in lanes 1 and 4. All DNA samples were electrophoresed through 1% agarose gels at 160V for 20 min stained with EtBr, and visualised under UV light.

Appendix E: Temperature gradient experiment for PCR optimisation

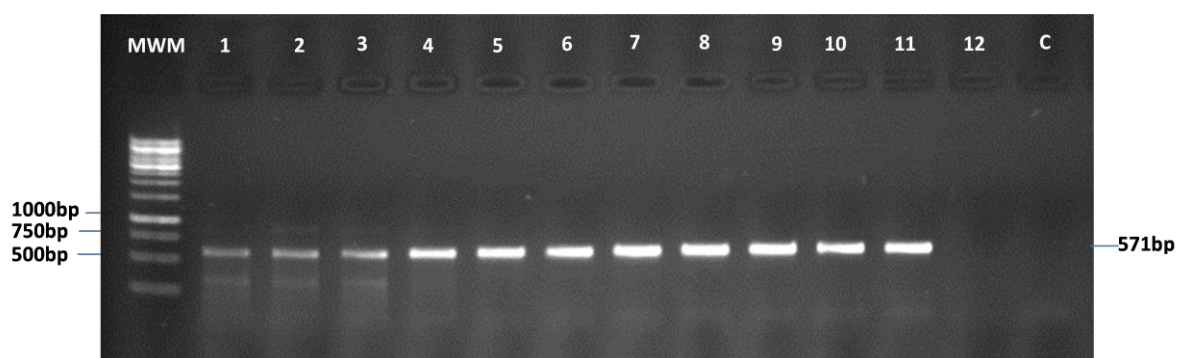
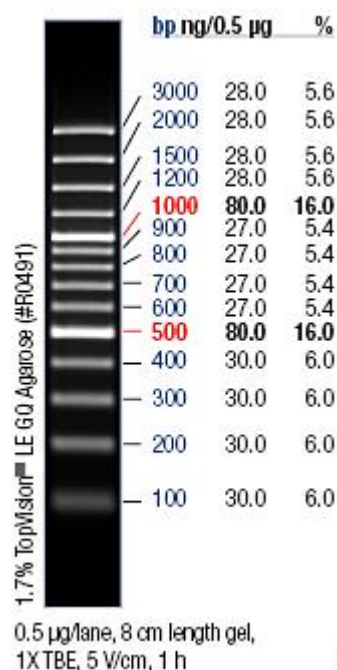


Figure B: Agarose gel electrophoresis of a temperature gradient experiment used for PCR optimisation of rs1045280 in *ARRB2*. MWM indicates a 1kb molecular weight marker: GeneRuler™ 1kb DNA Ladder Plus (Fermentas Life Sciences, Burlington, Canada), C indicates a no template control. The annealing temperatures are as follows: 1 - 48°C, 2 - 48.7°C, 3 - 49.3°C, 4 - 50.9°C, 5 - 52.7°C, 6 - 54.4°C, 7 - 55.2°C, 8 - 56.7°C, 9 - 58.1°C, 10 - 59.3°C, 11 - 59.6°C, 12 - 60°C. PCR products were electrophoresed through 1% agarose gels stained with EtBr at 160V for 40 min, and visualised under UV light.

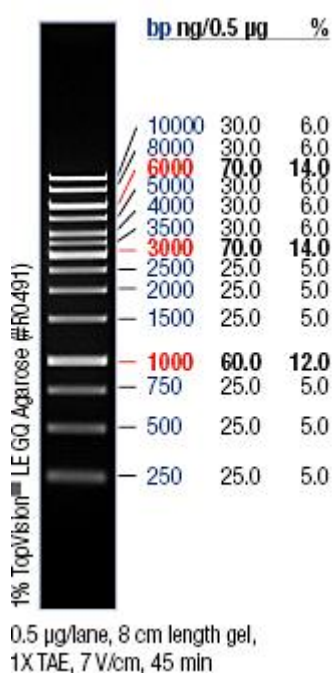
Appendix F: Molecular Weight Markers and Size Standards

GeneRuler™ 100 bp DNA Ladder Plus (Fermentas Life Sciences, Burlington, Canada)



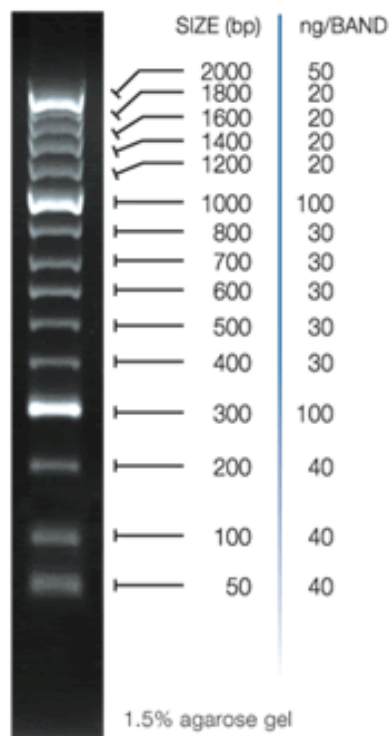
(Adapted from: <http://www.thermoscientificbio.com/nucleic-acid-electrophoresis/generuler-100-bp-plus-dna-ladder-ready-to-use-100-to-3000-bp/>)

GeneRuler™ 1kb DNA Ladder Plus (Fermentas Life Sciences, Burlington, Canada)



(Adapted from: <http://www.thermoscientificbio.com/nucleic-acid-electrophoresis/generuler-1-kb-dna-ladder-ready-to-use-250-to-10000-bp/>)

HyperLadder™ 50bp (Bioline, UK)



Adapted from: (http://www.bioline.com/h_prod_detail_ld.asp?itemid=152)

Appendix G: Reagents, Buffers and Solutions

10X Tris-borate-EDTA (TBE) buffer (stock)

- 108 g tris (hydroxymethyl) aminomethane
- 55 g boric acid
- 7.4 g ethylenediaminetetra-acetic acid (EDTA)

Dissolve and make up to a final volume of 1 litre with distilled water. Dilute as required.

1x TBE Buffer (working stock)

1:10 dilution was made with stock TBE buffer using dH₂O.

6x DNA Loading dye (Fermentas Life Sciences, Burlington, Canada)

- 10 mM Tris-HCl (pH 7.6)
- 0.03% bromophenol blue
- 0.03% xylene cyanol FF
- 60% glycerol
- 60 mM EDTA

Dilute to 1x with distilled water.

1% Agarose gel

- 1 g SeaKem® LE Agarose (Lonza, USA)
- 100 ml 1x TBE buffer
- 6 µl (5 ng/µl) ethidium bromide (Sigma, England)

2% Agarose gel

- 2 g SeaKem® LE Agarose (Lonza, USA)
- 100 ml 1x TBE buffer
- 6 µl (5 ng/µl) ethidium bromide (Sigma, England)

4% Agarose gel

- 4 g SeaKem® LE Agarose (Lonza, USA)
- 100 ml 1x TBE buffer
- 6 µl (5 ng/µl) ethidium bromide (Sigma, England)

Appendix H: General Protocols

Standard PCR reaction mixture

Reagent	Stock Concentration	Required concentration/amount	Volume
Forward Primer	20 μ M	10 pmol	0.5 μ l
Reverse Primer	20 μ M	10 pmol	0.5 μ l
dNTPs (Bioline, UK)	5 mM	200 μ M	1 μ l
Colorless GoTaq reaction buffer (Promega, USA)	5x	1x	5 μ l
GoTaq DNA polymerase (Promega, USA)	10 U/ μ l	1 U	0.1 μ l
Sterile distilled H ₂ O			16.9 μ l
Template DNA	100 ng/ μ l	100 ng	1 μ l
Total Volume			25 μ l

Reagents used in the SNaPshot PCR genotyping reaction

Reagent	Singleplex	Multiplex
Cleaned-up PCR product	1 μ l	5 μ l
SNaPshot™ Multiplex Ready Reaction Mix	1 μ l	1 μ l
Primers	1 μ l	1 μ l (pooled)
dH ₂ O	7 μ l	3 μ l
Total Volume	10 μ l	10 μ l

Ethanol Precipitation following cycle sequencing

1. Transfer cycle sequencing reaction product to a 1.5ml eppendorf tube.
2. Add 50 μ l chilled 100% ethanol and 2 μ l chilled sodium acetate
3. Vortex to mix and hold at -20°C for 2 hours
4. Centrifuge at 10 000rpm for 10 min and discard the supernatant
5. Add 30 μ l chilled 70% ethanol and vortex to mix
6. Centrifuge at 10 000rpm for 10 min
7. Air dried for approximately 1 hour to evaporate ethanol
8. Resuspend in dH₂O.

Appendix I: Correlations between side effects

Table A: Correlations between twenty side effects investigated in this study. Abbreviations: Corr: Correlation (r^2 value); Sig.: p-value; N: number of patients; Nau: Nausea; Dia: Diarrhoea; Trem: Tremor; Polyd: Polydipsia; Polyu: Polyuria; Mem: Memory and Concentration problems; Bod: Bodyache; Drow: Drowsiness; Cons: Constipation; Indi: Indigestion; Dry: Dry Mouth; Head: Headache; Stuf: Stuffy Nose; Wei: Weight gain; Met: Metallic Taste; App: Poor appetite; Diz: Dizziness/Giddiness; Blur: Blurred vision; Thy: Thyroid problems; Skin: Skin problems

		Nau	Dia	Trem	Polyd	Polyu	Mem	Bod	Drow	Cons	Indi	Dry	Head	Stuf	Wei	Met	App	Diz	Blur	Thy	Skin
Nau	Corr	1	.220*	.281**	.257*	.228*	.000	.225*	.236*	.082	.323**	.145	.154	-.019	.066	.166	-.060	.159	.218*	.121	.141
	Sig.		.028	.005	.010	.027	.998	.024	.017	.413	.001	.151	.125	.853	.513	.099	.549	.111	.029	.229	.161
	N	101	100	100	99	94	100	101	101	101	101	99	101	101	100	100	101	101	101	100	101
Diar	Corr	.220*	1	.146	.140	.195	.067	.250*	.090	.063	-.014	.198*	-.002	-.028	.043	.016	.071	.033	.196*	.042	-.038
	Sig.	.028		.144	.164	.057	.502	.011	.366	.526	.888	.047	.986	.779	.669	.871	.478	.741	.047	.672	.700
	N	100	103	102	100	96	102	103	103	103	103	101	103	103	102	102	103	103	103	102	103
Trem	Corr	.281**	.146	1	.206*	.102	.015	.135	.026	.076	.233*	.221*	.209*	.132	.125	.226*	-.082	.101	.173	-.107	-.068
	Sig.	.005	.144		.038	.321	.881	.171	.790	.444	.017	.026	.033	.182	.207	.022	.406	.309	.080	.281	.494
	N	100	102	104	101	96	103	104	104	104	104	102	104	104	103	103	104	104	104	103	104
Polydip	Corr	.257*	.140	.206*	1	.563**	-.068	.191	.086	.203*	.070	.535**	.143	.157	.150	.086	.051	.157	.163	.055	.028
	Sig.	.010	.164	.038		.000	.498	.054	.389	.040	.482	.000	.153	.114	.134	.393	.609	.115	.101	.584	.777
	N	99	100	101	102	96	101	102	102	102	102	101	102	102	101	101	102	102	102	101	102
Polyu	Corr	.228*	.195	.102	.563**	1	-.067	.100	.361**	.137	.057	.381**	.036	.178	.212*	.058	.243*	.111	-.030	.070	.007
	Sig.	.027	.057	.321	.000		.516	.332	.000	.180	.581	.000	.727	.081	.038	.572	.016	.280	.767	.498	.946
	N	94	96	96	96	97	96	97	97	97	97	96	97	97	96	96	97	97	97	96	97
Mem	Corr	.000	.067	.015	-.068	-.067	1	.130	.162	.111	-.031	.199*	-.075	-.066	.066	.122	.231*	.245*	.217*	-.042	-.142
	Sig.	.998	.502	.881	.498	.516		.189	.099	.261	.758	.045	.448	.506	.508	.220	.018	.012	.027	.674	.149
	N	100	102	103	101	96	104	104	104	104	104	102	104	104	103	103	104	104	104	103	104
Bod	Corr	.225*	.250*	.135	.191	.100	.130	1	.046	.040	.193*	.153	.185	.360**	.162	.166	-.031	.356**	.363**	.012	.080
	Sig.	.024	.011	.171	.054	.332	.189		.644	.686	.048	.123	.058	.000	.100	.093	.752	.000	.000	.903	.415
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105
Drow	Corr	.236*	.090	.026	.086	.361**	.162	.046	1	-.059	.115	.183	.030	-.020	.273**	-.075	.060	.033	.237*	.165	.014
	Sig.	.017	.366	.790	.389	.000	.099	.644		.549	.244	.065	.762	.841	.005	.447	.542	.736	.015	.094	.888
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105
Cons	Corr	.082	.063	.076	.203*	.137	.111	.040	-.059	1	.349**	.115	.029	.136	-.097	.020	-.077	.135	-.060	.148	-.054
	Sig.	.413	.526	.444	.040	.180	.261	.686	.549		.000	.246	.770	.168	.327	.842	.435	.169	.546	.133	.585
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105
Indi	Corr	.323**	-.014	.233*	.070	.057	-.031	.193*	.115	.349**	1	.033	.290**	.105	.027	.182	-.016	.224*	.121	.035	.130
	Sig.	.001	.888	.017	.482	.581	.758	.048	.244	.000		.738	.003	.286	.783	.064	.871	.022	.217	.721	.185
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105

		Nau	Dia	Trem	Polyd	Polyu	Mem	Bod	Drow	Cons	Indi	Dry	Head	Stuf	Wei	Met	App	Diz	Blur	Thy	Skin
Dry	Corr	.145	.198*	.221*	.535**	.381**	.199*	.153	.183	.115	.033	1	.185	.105	.246*	.230*	.007	.201*	.097	.199*	.059
	Sig.	.151	.047	.026	.000	.000	.045	.123	.065	.246	.738		.061	.292	.013	.020	.943	.042	.328	.045	.554
	N	99	101	102	101	96	102	103	103	103	103	103	103	103	102	102	103	103	103	102	103
Head	Corr	.154	-.002	.209*	.143	.036	-.075	.185	.030	.029	.290**	.185	1	.313**	.269**	.222*	.116	.004	.242*	-.028	.320**
	Sig.	.125	.986	.033	.153	.727	.448	.058	.762	.770	.003	.061		.001	.006	.023	.239	.969	.013	.779	.001
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105
Stuf	Corr	-.019	-.028	.132	.157	.178	-.066	.360**	-.020	.136	.105	.105	.313**	1	.044	.153	.111	.145	-.007	-.068	.115
	Sig.	.853	.779	.182	.114	.081	.506	.000	.841	.168	.286	.292	.001		.660	.120	.259	.139	.946	.495	.243
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105
Wei	Corr	.066	.043	.125	.150	.212*	.066	.162	.273**	-.097	.027	.246*	.269**	.044	1	.215*	.203*	.120	.179	.112	.071
	Sig.	.513	.669	.207	.134	.038	.508	.100	.005	.327	.783	.013	.006	.660		.029	.039	.224	.068	.259	.473
	N	100	102	103	101	96	103	104	104	104	104	102	104	104	104	103	104	104	104	104	104
Met	Corr	.166	.016	.226*	.086	.058	.122	.166	-.075	.020	.182	.230*	.222*	.153	.215*	1	.225*	.344**	.224*	.012	.176
	Sig.	.099	.871	.022	.393	.572	.220	.093	.447	.842	.064	.020	.023	.120	.029		.022	.000	.022	.902	.074
	N	100	102	103	101	96	103	104	104	104	104	102	104	104	103	104	104	104	104	103	104
App	Corr	-.060	.071	-.082	.051	.243*	.231*	-.031	.060	-.077	-.016	.007	.116	.111	.203*	.225*	1	.005	.005	-.031	-.028
	Sig.	.549	.478	.406	.609	.016	.018	.752	.542	.435	.871	.943	.239	.259	.039	.022		.960	.959	.753	.779
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105
Diz	Corr	.159	.033	.101	.157	.111	.245*	.356**	.033	.135	.224*	.201*	.004	.145	.120	.344**	.005	1	.260**	.142	.168
	Sig.	.111	.741	.309	.115	.280	.012	.000	.736	.169	.022	.042	.969	.139	.224	.000	.960		.007	.151	.087
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105
Blur	Corr	.218*	.196*	.173	.163	-.030	.217*	.363**	.237*	-.060	.121	.097	.242*	-.007	.179	.224*	.005	.260**	1	.108	.245*
	Sig.	.029	.047	.080	.101	.767	.027	.000	.015	.546	.217	.328	.013	.946	.068	.022	.959	.007		.274	.012
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105
Thy	Corr	.121	.042	-.107	.055	.070	-.042	.012	.165	.148	.035	.199*	-.028	-.068	.112	.012	-.031	.142	.108	1	.344**
	Sig.	.229	.672	.281	.584	.498	.674	.903	.094	.133	.721	.045	.779	.495	.259	.902	.753	.151	.274		.000
	N	100	102	103	101	96	103	104	104	104	104	102	104	104	104	103	104	104	104	104	104
Skin	Corr	.141	-.038	-.068	.028	.007	-.142	.080	.014	-.054	.130	.059	.320**	.115	.071	.176	-.028	.168	.245*	.344**	1
	Sig.	.161	.700	.494	.777	.946	.149	.415	.888	.585	.185	.554	.001	.243	.473	.074	.779	.087	.012	.000	
	N	101	103	104	102	97	104	105	105	105	105	103	105	105	104	104	105	105	105	104	105

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).